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Blood Substitutes: General

Guest Editors:
T.M.S. Chang, J.G. Riess, and R.M. Winslow

ARTIFICIAL CELLS, BLOOD SUBSTITUTES, AND IMMOBILIZATION BIOTECHNOLOGY

May 1994

Aims and Scope. This journal covers artificial cells, blood substitutes, and immobilization biotechnology. The emphasis of this journal is to allow for interdisciplinary interactions. Therefore, we welcome approaches based on biotechnology, chemical engineering, medicine, surgery, biomedical engineering, basic medical sciences, chemistry and others. The following areas are particularly welcomed.

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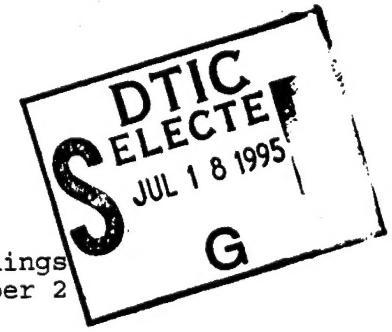

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BLOOD SUBSTITUTES: GENERAL

First of 3 Special Issues (Peer Reviewed) from
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San Diego, California, USA
March 17-20, 1993

Guest Editors

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ARTIFICIAL CELLS, BLOOD SUBSTITUTES, AND IMMOBILIZATION BIOTECHNOLOGY

Volume 22, Number 2, 1994

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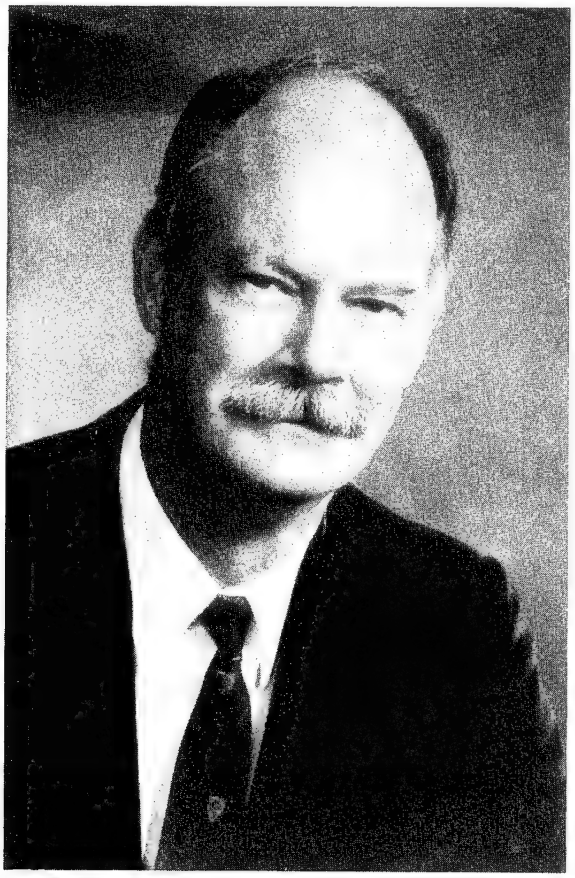
DEDICATION

(transcribed from Dr. Winslow's opening remarks at the Vth International Symposium on Blood Substitutes)



Dr. John Collins

Before officially opening the Vth International Symposium on Blood Substitutes, I would like to take a moment to pay tribute to a colleague and friend who is not with us. Dr. John Collins, tragically, died prematurely on September 21, 1992, while with his wife, Maureen, in Ireland. He was 58 years old.

I am not sure whether John actually would have been here with us -- he viewed most of us interested in blood substitutes as being close to the lunatic fringe. But even though many of you did not have the privilege to know John, his presence has been felt by all of us.

John was born in New York City on October 23, 1933 and was educated in New York. His AB degree was from Fordham, and his MD was from Columbia. He served his internship at Columbia-Presbyterian Hospital in New York, and his residency was at Mary Imogene Bassett Hospital in Cooperstown, New York.

John had an outstanding career. He held the ranks of Assistant, Associate, and full Professor at the Washington University School of Medicine before becoming Chairman of the Department of Surgery, Stanford University School of Medicine in 1977, a post he held until his death. He authored approximately 126 scientific papers, and served on editorial boards of several prestigious journals including Surgery and the American Journal of Surgery. He was also an outstanding teacher: he received numerous awards such as "Teacher of the Year" at Washington University School of Medicine and the Kaiser Award for Outstanding Contributions to Teaching at Stanford.

Very significantly, John served in the Medical Corp of the U.S. Army between 1965 and 1967 with a rank of Captain. He was Chief of the Department of Human Studies, Division of Surgery, Walter Reed Army Institute of Research. Then, from April to October, 1966, he was Chief of the Army Surgical Research Team in Vietnam. I am sure this experience contributed to his very thoughtful approach to resuscitation and fluid therapy.

In 1991, John chaired an NIH Workshop on the Evaluation of Plasma Expanders. The proceedings of that workshop, when published soon in the Archives of Surgery, will be typical of the type of contribution he made. That is, he was almost unique in his ability to sift through a mass of contradictory and inconsistent data and reduce it to something comprehensible.

I was fortunate to serve with John on two Government panels. The first was the FDA/Army/NIH conference held on March 14, 1990, to evaluate the state of development of Blood Substitutes. This meeting produced the now-famous "Points to Consider in the Development of Hemoglobin-based Oxygen Carriers".

The second was the Naval Research Advisory Committee Panel on "Delivery of Artificial Blood to the Military". The work of this panel was just finished when John died last fall. It was in the course of the work of this panel that I came to really appreciate John Collins. His attitude was that of the fascinated skeptic. Although he and I disagreed on many points during the deliberations, the disagreements were always on a scientific, not personal, level, and were resolved by rational (but on occasion, animated) discussions.

In the end, John was excited by the prospects of a new generation of artificial oxygen carriers, and I know he was in the process of planning experimental work with some of them when he died. He forcefully argued that we need to improve the quality of science in this field and, he felt that many of the commonly-used animal models are not adequate to evaluate new products.

John Collins shared my conviction that the only way we can progress toward the goal of developing clinically-useful products is to communicate with each other about our data and our ideas. And so we are here to begin what I know will be a remarkable Symposium. In my view, the quality of lectures and posters is extremely high. We have a marvelous environment and a feast of new data and ideas to discover and discuss with one another.

ART. CELLS, BLOOD SUBS., AND IMMOB. BIOTECH., 22(2), xvii (1994)

ACKNOWLEDGEMENTS

The chairpersons of a symposium such as this one customarily receive the blame for all organizational and logistical problems. We accept all responsibility for such mishaps and hope they have been few. The Chairpersons also usually receive any praise or crdit to be given. In the case of the Vth ISBS, however, this is inappropriate because any success we may have had in preparing for and executing the detailed plans of the meeting is clearly due to a number of other people. They included Renée Schad, of the University of California, San Diego, and Shirley Kolkey of Complete Conference Management, who contributed enormously to all phases of the meeting and its' success. Valérie Briet, Phil Calvillo, Armando Gonzales, Marie-Pierre Krafft, Wava McCluskey, and Gwen Rosenberg also skillfully handled many of the myriad details which were required, not only during the meeting, but also during the weeks and months before it. We also thank those members of the International Committee for Blood Substitutes who have contributed proposals and suggestions to the symposium. Finally, we wish to express thanks to the staff of the Sheraton Harbor Island Hotel who helped make this meeting not only a success, but a comfortable pleasure.

Thomas M.S. Chang, O.C.,M.D. *Jean G. Riess, Ph.D.* *Robert M. Winslow, M.D.*

GENERAL PLENARY LECTURES

ART. CELLS, BLOOD SUBS., AND IMMOB. BIOTECH., 22(2), 123-135 (1994)

OXYGEN CARRIERS AND TRANSFUSION MEDICINE

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ABSTRACT

The US blood supply is once again expanding (14 million units a year) and annual estimated whole blood and red blood cell (RBC) transfusion now exceeds 12 million units. The observed increase in total transfusions and units transfused per surgical procedure may result from more aggressive therapies, an aging population, and improved access to health care. While autologous blood collection has grown 20-fold in the past decade, autologous blood still accounts for <8% of transfusions and is unlikely to replace much more of the allogeneic transfusion needs. Although safer than ever, allogeneic blood still transmits infectious disease (HIV:1 in 225,000 units, hepatitis:1 in 3300 units, HTLV I/II:1 in 50,000 units) and poses additional immunologic and non-immunologic risks. Allogeneic RBCs are probably underutilized because of safety concerns.

While the cost of a unit of RBCs has been estimated at $150, costs are substantially higher in some areas and blood processing (filtration, gamma irradiation, washing) add additional expense. The narrowing margin between supply and demand, and repeated regional blood shortages argue for the value of safe, effective oxygen carriers.

The last two decades have witnessed remarkable changes in the growth, use, and public perception of blood transfusion in the United States. National surveys indicate that blood collections and transfusions doubled between 1971 and 1980 [1]. National Blood Policy, formulated in the early 1970's, outlined principles for an adequate, safe, available, and affordable blood supply; the national commitment to all-volunteer blood donation, coupled with the use of sensitive screening tests for hepatitis B virus (HBV), promised to make these goals achievable. By 1980, blood collectors, medical practitioners, most policy makers, and the general public were largely satisfied with the apparent progress in securing a safe and available blood supply.

By 1984 the situation had changed dramatically. Epidemiologic evidence had linked blood transfusion with a new and frightening fatal illness, the acquired immune deficiency syndrome (AIDS) [2]. Non-specific screening techniques that were introduced to improve blood safety by eliminating donors with high risk behavior had deferred large numbers of long-time donors. By 1985, as many as 34 percent of blood donors in one survey believed that the blood donation process itself likely involved a risk of contracting AIDS [3]. While mass media concerns about blood safety increasingly centered

on AIDS, the medical community became concerned simultaneously about the risk of hepatitis. The recognition of transfusion-transmitted hepatitis caused by virus(es) other than HBV led to the widespread and appropriate introduction of non-specific screening tests that eliminated 3 to 5% of American blood donors, most of these safe and healthy volunteers [4,5]. Transfusions of whole blood and red blood cells, which had peaked at 12.2 million units in 1986, declined to 11.6 million units in 1987 and collections of allogeneic blood reached a plateau at 13.3 million units [6]. By the end of the decade, public confidence in the volunteer blood supply had fallen to the extent that many otherwise reasonable and medically informed patients were accepting blood only from friends or relatives or refusing allogeneic transfusion altogether.

Although it is treacherous to divine transfusion trends, several themes are now emerging. Blood transfusion is in fact increasing once more. The total US blood supply in 1989 was 14,229,000 units, an expansion of 1.2 percent over collections in 1987 [7]. A portion of this increase resulted from autologous donations. While estimates from a 1986 survey suggested that no more than 5 percent of eligible donors were pre-depositing autologous blood for elective surgery, pre-deposited autologous collections increased by 65 percent between 1987 and 1989 alone and represented 4.6 percent of the blood supply in 1989 [7,8]. Various less well controlled estimates place current autologous collections as high as 8 percent of donated units; however, autologous units are unlikely to far exceed 10 percent of total blood collections.

Another source of growth in the American blood supply has been directed donations, blood solicited by patients from friends and relatives. Directed donations accounted for 2.5 percent of the blood supply in 1989. Directed donors must meet all the criteria required of volunteer community donors, however, like autologous blood collected from patient-donors, directed donor units have been found to test positive more frequently for markers of such infectious diseases as hepatitis [9]. The safety for general use of blood from patients and directed donors has been the subject of heated controversy [10]. Although both autologous and directed units, when appropriately collected and tested, may be "crossed over" into the general blood supply (46.7 percent of the autologous units and 55.4 percent of the directed units are not transfused), only 2 percent of autologous and 16.9 percent of directed units end up supplementing the national blood supply [7].

One source of blood for transfusion that is admittedly underestimated is intraoperative autologous blood (IAT), blood salvaged from the operative field and returned to the patient during the surgical procedure. No single reliable source collects and reports the amount of blood salvaged by IAT procedures. IAT is performed by regional blood centers, hospital transfusion services, anesthesia technicians, surgical support groups, and private for-profit companies. IAT is usually restricted to procedures such as cardiac, vascular, orthopedic and transplant surgery where large intraoperative blood loss is anticipated, and to emergency trauma surgery. However in these relatively

limited situations, autologous salvage may replace large volumes of blood, 100 units or more in some procedures [11]. Massive IAT requires expensive equipment, trained technical support and costly plastic disposable software.

Why is red blood cell transfusion increasing, will this trend continue, and will the volunteer blood supply be able to meet projected needs? None of these questions has well-documented answers. The most likely explanations include the following: (a) The American population is aging, and elderly patients require more hospitalizations, more surgical procedures, and more transfusions. (b) More sophisticated medical and surgical procedures, including aggressive cancer chemotherapy, organ and marrow transplantation, and anti-viral AIDS therapy depend on transfusion support. (c) As the national network of trauma centers develops, major trauma consumes an increasing number of red blood cell units, particularly group O red blood cells. (d) More Americans are demanding access to medical care and federal health planners appear committed to providing the means for such access. Despite the increase in autologous and directed units, the growth in IAT, the increase in imports of European red blood cells, and the suspected elimination of much of the frivolous use of red blood cell transfusion, segmentation of the national blood resource suggests that by 1989 the margin between blood supply and demand had become perilously thin [7].

Ironically, allogeneic blood is safer today than it has ever been. The major public concern, the risk of transfusion-transmitted viral infection, has been reduced extraordinarily [12,13]. The risk of HIV from

transfusion is now estimated at one case per 225,000 units transfused. Fewer than 25 cases have been reported since specific anti-HIV testing was introduced in March of 1985. The risk of hepatitis B is negligible, an estimated one case in 200,000 units transfused. Hepatitis from transfusion is estimated at about one case per 3300 units transfused. A variety of other infectious agents, viral parasitic, and bacterial, are thankfully rare in the United States. Nevertheless until cellular blood components can be sterilized, patients will have little choice but to accept the relatively low risk of known infectious agents, and the unsettling concern that some as yet unrecognized infectious agent might threaten either the safety or availability of blood. As an example of the latter concern, the recognition of a small number of infections with the obscure parasite, Leishmania tropicalis, among American forces serving in the middle east during Operation Desert Storm, led to a temporary deferral for one year of 500,000 potential blood donors.

There are other recognized risks of allogeneic blood transfusion. Minor reactions, fever, chills, and dermatologic reactions, complicate about 1 percent of transfusions. These reactions are of little clinical consequence. However, even minor reactions cause distress to patients and generally trigger laboratory and clinical investigations to eliminate more serious causes of these findings. Hemolytic transfusion reactions complicate about one in 6000 units transfused and fatal reactions may be as frequent as one in 100,000 transfusions. These complications result from the need to provide red blood cells that are

serologically compatible with the recipient's blood. Mounting evidence implicates allogeneic blood transfusion as a modulator of immune changes in the transfusion recipient. A variety of laboratory abnormalities, suggesting alterations of the immune response, have been reported after allogeneic blood transfusion [14]. Clinical correlations have been less apparent. Nevertheless, transfusions have long been used to suppress the recipient's immune response to transplanted kidneys, and numerous reports suggest that patients who receive allogeneic blood during surgery suffer an increased number of postoperative infections compared to patients who receive no transfusion or who receive autologous blood only [15]. Furthermore, patients who undergo surgery for a variety of different tumors, including colon carcinoma, soft tissue sarcoma, lung, breast, and prostate cancer, reportedly have decreased survival and shorter tumor-free survival if they receive allogeneic blood in the perioperative period. Although none of these studies is conclusive and although conflicting evidence has been published [16], no study shows a beneficial effect of transfusion in regard to infections, tumor-free survival, or overall survival from cancer.

Perhaps most distressing are recent reports that allogeneic blood transfusions may reactivate latent viral infections in the recipient [17]. Laboratory investigators have long recognized that allogeneic stimulation of cells in vitro can result in activation and spread of both cytomegalovirus (CMV) and HIV in cell culture systems [18]. Studies have implicated allogeneic transfusion as stimulating reactivation of

CMV in bone marrow transplant recipients and in adversely affecting the survival of patients with AIDS [19]. If, as some reports suggest, the mechanism of activation depends upon leukocytes that contaminate red blood cell transfusions, the removal of such cells will at the very least increase the cost of blood transfusion. If the mechanism involves some intrinsic property of the red blood cell, an important and difficult hazard of allogeneic transfusion will need to be addressed.

Estimates of the projected applications of an oxygen carrier as a synthetic blood substitute generally start with the current number of red blood cell units transfused, especially in emergency situations and in the perisurgical setting. Such estimates are clearly conservative. A desire on behalf of both patient and physician to limit the risk of blood transfusion has led to increasingly lowered recommendations for the "transfusion trigger," the hemoglobin value at which transfusion is indicated [20]. In some clinical settings, insistence on the lowest possible hemoglobin concentration as a threshold for transfusion may impair tissue oxygenation and threaten patient safety [21]. Were the risks and perceived risks of transfusion not a factor, there would be little need to titer the hemoglobin concentration so carefully. A safe, effective oxygen carrier might provide a wider margin of safety for patients with impaired cardiovascular and pulmonary compensatory mechanisms.

If superior safety and availability provide a powerful rationale for developing synthetic oxygen carriers, economic considerations have also stimulated

recent research and development efforts. A study of 19 hospitals in four geographic regions estimated the cost of delivering a unit of whole blood or red blood cells in 1989 at $155 [22]. This cost estimate was based on an average cost of blood procurement from regional blood centers ($52 or 37 percent of total hospital blood costs) and the additional costs of handling, testing, and administering blood. Investigators at a single university medical center used different methodology, including estimated posttransfusion costs, such as the cost of contracting an infectious disease from transfusion or the cost of developing a transfusion reaction, to estimate that a unit of red blood cells provided for a surgical patient costs $149.25 (DA Lubarsky, Duke University Medical Center, personal communication). Both probably underestimate the average cost of a unit of blood in 1993. Even at current levels of transfusion for urgent surgery and trauma, red cell transfusion represents a multi-billion dollar expense for American health care.

Historic trends suggest that the cost of providing red blood cells is unlikely to plateau or decrease in the foreseeable future. Hospitals with more than 55 beds paid $32 to acquire a unit of blood and charged $84 in 1979, compared to the estimates of $155 and $219 calculated in 1989 [22]. Increased donor selectivity continues to drive up the costs of recruitment; additional laboratory screening tests can be anticipated; increased regulatory oversight may add expense for both the regional blood collector and for the hospital. If fewer components, such as platelets, plasma and cryoprecipitate, are separated from units of

whole blood, the cost of the red blood cells will inevitably rise. The growth of single donor platelets and the availability of virus-free clotting factors suggest that fewer "by-product" components may be needed. In addition, more red blood cell units require specialty processing such as leukocyte reduction and gamma irradiation. Twenty-one percent of the units reported by Forbes required such preparation, which added 33 percent to their cost; autologous and directed "specialty donations" added 50 percent [22]. Finally, costs of litigation may add substantial cost. A single award in 1990 had the effect on the health care system of adding one dollar to the cost of every unit transfused in the United States during that year [23].

The desirability of a synthetic red blood cell substitute seems obvious. Although optimists may argue that the narrowing margin between collections and transfusions represents more efficient management of the national blood resource, more likely this slender safety margin presages a return to the era of regional blood shortages. The need for oxygen carriers is still increasing and is likely to do so for the next decade. Autologous collections have cushioned some of the loss of allogeneic units, but the percentage of eligible patients donating autologous blood is approaching predicted levels. Strategies such as marrow stimulation with recombinant human erythropoietin have been demonstrated to increase predeposit collections by up to 41 percent, however such strategies will benefit a limited subset of patients, those who require between 4 and 6 units of blood for an elective procedure that permits several weeks for blood collection [24]. While

erythropoietin has already been a major boon to patients with chronic renal disease and some patients with cancer and AIDS, it will probably have little further impact on the nation's blood supply. Intraoperative salvage technology has matured during the past decade, and while IAT remains a valuable adjunct to surgical support, it has technical and economic drawbacks. An effective, safe, and sterile oxygen carrier that is stable at room temperature or at refrigerated storage would provide a major advance in transfusion therapy.

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HEMODILUTION AND BLOOD SUBSTITUTES

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ABSTRACT

The primary consequence of the substitution or replacement of blood with a surrogate is the dilution of the original constituents. This hemodilution produces systemic and microvascular phenomena that underlie all forms of blood replacement and provides a physiological reference for comparison for blood substitutes. The basic features of hemodilution become evident when the procedure is carried out in isovolemic and isoocotic conditions where blood viscosity and oxygen carrying capacity are changed. Blood viscosity is decreased, which redistributes macro and microcirculatory blood pressure increasing the arterio/venular pressure difference and central venous pressure, which improves cardiac filling and cardiac output and therefore blood flow velocity. These effects coupled to the oxygen carried by the diluted blood maintains the rate of oxygen delivery to the microcirculation up to hematocrit reductions of one third. The increased flow velocity counteracts the diffusive losses of oxygen from the microvessels. The increased flow velocity increases shear stress at the vessel wall lowering the tendency of activated leukocytes to adhere. Hemodilution with dextran 70 also maintains functional capillary density to hematocrit decreases of one half. Hemodilution with $\alpha\alpha$-hemoglobin presents all the features found with non-oxygen carrying colloids, however the increased oxygen carrying capacity is not fully exploited because systemic and microvascular effects due to colloids do not develop to the same extent.

INTRODUCTION

The replacement or restitution of blood with an artificial material produces hemodilution, which is a well established process for decreasing the viscosity of blood. This procedure is usually made in isooncotic and isovolumetric conditions, in such a fashion that the homeostatic mechanisms that detect changes in blood volume are not activated. Thus most forms of blood substitution or replacement have as an objective the maintenance of normovolemia. The variables that can be manipulated in the selection of transport properties for the fluid with which to implement the substitution are the oncotic pressure, the viscosity and the oxygen carrying capacity of the mixture of original blood and substitute.

Isovolemic and isoocotic hemodilution with colloids circumscribes the changes in transport properties to the alteration of viscosity and intrinsic oxygen carrying capacity of the circulating mixture and provides a physiological reference with which to compare other types of blood substitution.

The end point of any alteration of the transport properties of blood is whether tissue metabolism is sustained, i.e., whether the tissue is adequately oxygenated, a phenomenon that takes place in the microvasculature. In this context hemodilution must be analyzed not only in terms of systemic effects but also in terms of how these, coupled with the altered composition of blood, influence the transport properties of the microcirculation.

TRANSPORT FACTORS IN HEMODILUTION

Hematocrit and Blood Viscosity

Blood viscosity is primarily determined by the hematocrit in the larger vessels while it is a weaker function of the systemic hematocrit in the microcirculation. Utilizing equations derived by Dintenfass [1], and Quemada [5], it can be shown that

at a given shear rate blood viscosity is approximately proportional to the hematocrit squared and inversely proportional to shear rate according to the relationship:

$$\eta = a_s + b_s H^2$$

while Lipowsky et al. [7] have shown that microvascular blood viscosity can be empirically described by a relation of the form:

$$\eta = a_m + b_m H$$

where η is the blood viscosity in centipoise and a_i's and the b_i's are parameters that are shear rate and vessel size dependent. It should be noted that in the microcirculation blood viscosity is relatively insensitive to shear rate.

Considering the functional difference between the hematocrit dependence that exist between macro and microcirculation it is apparent that when hematocrit is reduced systemic viscous pressure losses will decrease much more rapidly than those in the microvasculature, while in the microcirculatory the A-V pressure drop is not very much affected. The net result is that if arterial pressure remains constant, hemodilution produces a significant pressure re-distribution in the circulation as shown by Mirhashemi et al. [14]. Given that the majority of experience with hemodilution validates the constancy of systemic pressure during this procedure, the pressure redistribution is a logical consequence, although one that has not been verified experimentally in the microcirculation.

An important systemic pressure effect produced by the lowered hematocrit and blood viscosity occurs in the venous return where central venous pressure is increased, which improves cardiac performance and increases cardiac output [16]. In a vasculature that remains at constant intravascular volume, this effect translates into an increased blood flow velocity and shear rate and therefore lower blood viscosity in the systemic circulation.

Hemodilution and Oxygen Transport

The decrease in hematocrit lowers the intrinsic oxygen carrying capacity of blood, but this effect is compensated by the increased blood flow velocity which increases the rate at which the oxygen carrying red blood cells are delivered to the microcirculation. The net result is that both the systemic as well as the capillary oxygen carrying capacities remain approximately constant down to arterial hematocrits of the order of 25%. This phenomenon was first demonstrated by Hint, [4], and has been verified by Messmer et al. [11] in the systemic circulation and by Mirhashemi et al. [13], Lipowsky and Firrel [8] and Tsai et al. [17] in the microcirculation.

Both theoretical and experimental studies of hemodilution support the concept that the reduction of hematocrit increases the oxygen delivery capacity of the microcirculation, however the improvement which occurs in the vicinity of hematocrit 33% is small and at most of the order of 10%. The effect is not sufficient to explain the improvement of tissue oxygenation found during hemodilution by means of direct measurement with multiwire microelectrodes [11].

The architecture of the microcirculation determines additional effects that support the maintenance of capillary oxygen delivery capacity. The principal barrier for the exit of oxygen from the blood vessels is its diffusion constant which is fairly uniform and of the same order as the diffusion constant of oxygen through water. This is valid for most the soft tissues including the blood vessel wall. As a consequence oxygen leaks out continuously from the blood column to the extent that upon arrival to the microcirculation virtually half of the oxygen gathered in the lung has been lost as shown by Duling and Berne [2]. The fact that venules are juxtaposed to the arterioles in a counter current configuration provides an additional mechanism for oxygen loss. The increase of blood flow velocity diminishes transit time and therefore leakage and shunting from the distributing arterioles and collecting venules, allowing more oxygen to reach the capillaries, and thus improving the utilization of the available oxygen.

Hemodilution and Leukocytes

The distribution of leukocytes in the cross section of the microvascular lumen is a flow dependant phenomenon, whereby as the velocity of the flow increases the leukocrit is highest in the blood cell rich core of arterioles and venules. This situation changes as velocity decreases causing the leukocytes to migrate toward the vessel wall [3] therefore with decreasing flow rate the possibility for endothelium-leukocyte interaction is enhanced.

The attachment of leukocytes to the vessel wall is the resultant of a balance between the adhesive forces generated at their activated surface and the stress imparted by the flowing blood. An increased shear stress proportionally lowers the number of adhering cells, which may be one of the factors responsible for the decrease in leukocyte adhesion noted when hemodilution is used prophylactically prior to an ischemic injury (Menger et al. [10] and Nolte et al. [15]).

Hemodilution and Functional Capillary Density

Tissue oxygenation is not only determined by the rate at which oxygen carriers are delivered to the microcirculation but also by the presence of a normal distribution system for these carriers. While there is a fraction of the oxygen that is delivered directly by the larger vessel vessels as evidenced by the decrease in pO_2 of blood as it nears the microcirculation, the primary mechanism of oxygen delivery is through the capillary system.

In the past it had been assumed that capillaries are essentially inert tubes (or endothelium lined tunnels) that unless adversely affected by thrombosis remain essentially open to the passage of plasma and the formed element in blood. Recent studies have shown that internal diameter of a capillary which cannot be smaller than 2.8 μm for the passage of red blood cells, may undergo significant changes as a consequence of the changes of volume of the endothelium and the state of hydration of the tissue [9].

The hydraulic conductivity of the capillaries may be additionally affected by the rigidity of the red blood cells and the activation of leukocytes. Several studies indicate that adequate capillary flow is dependant on a threshhold pressure gradient. This was shown directly in the microcirculation in the study of Lindbom and Arfors [6], where the gradual lowering of arterial pressure in skeletal muscle causes the number of flowing capillaries to decrease. This phenomenon was found to be reversible and when pressure was increased the capillaries were again perfused and in the same (but reverse) sequence in which they had stopped flowing.

When all the factors that determine tissue oxygenation are taken into account, the parameter m_{Oxygen} that characterizes the rate of oxygen delivery to the tissue from a microscopic basis may be defined be the following relationship:

$$m_{Oxygen} = Q\, C_{Oxygen}\, FCD$$

where Q is the capillary flow, C_{Oxygen} is the local oxygen content of circulating mixture of blood and substitute, and FCD is the functional capillary density, i.e., the number of capillaries in which there is through flow. Microvascular techniques allow to determine the changes that occur from given control conditions [18] and show that hemodilution with dextran 70 maintains FCD.

Hemodilution and Blood Substitutes

Data on blood substitutes relative to their effects as blood diluents in terms of microhemodynamic physiological considerations is limited. Our own studies (see paper by Tsai et al., this volume) tend to support the maintenance of oxygen carrying capacity by the substitution of blood with hemoglobin solutions, however there are important variations relative to colloids particularly in what regards the increase in blood flow velocity and the maintenance of functional capillary density.

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TRAUMA AND MILITARY APPLICATIONS OF BLOOD SUBSTITUTES

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ABSTRACT

PURPOSE: To review potential clinical uses of erythrocyte substitutes in treating military battlefield casualties, with specific emphasis on combat injury rates and wounding patterns, resuscitation doctrine and logistic requirements.
METHODS: Review of published medical literature and of unclassified documents from the U.S. Armed Forces Blood Program.
RESULTS: Hemorrhage is the leading cause of death on the battlefield. Early intervention, with definitive treatment, could save up to 30% of soldiers who are killed in action or who die of wounds. Hemorrhage control and rapid volume expansion in appropriate casualties are the main priorities in pre-hospital resuscitation of battlefield casualties. The role for oxygen-carrying fluids in the initial management of military injuries is undefined; however, erythrocyte substitutes could reduce the logistic requirements for blood in field hospitals. In recent wars, outdating of stored blood resulted in 60-95% of units being discarded: 60% of 1.3 million units in Vietnam and 95% of 120,000 units in the Persian Gulf War.
CONCLUSIONS: Safety, long storage life, light unit weight, and tolerance to environmental extremes are all characteristics that are necessary for erythrocyte substitutes to extend or replace the use of stored blood in treating battlefield casualties.

INTRODUCTION

Hemorrhagic shock is the leading cause of death in soldiers injured on the battlefield. In contrast to injuries that cause extensive destruction of the central nervous system or other vital organs, the lethal insult in hemorrhagic shock is blood loss. Hemorrhage results in depleted intravascular volume, decreased oxygen transport to tissues, and decreased removal of metabolic waste products. Replacing intravascular volume by fluid resuscitation is highly effective in treating hemorrhagic shock.

Crystalloid solutions expand the circulating blood volume, restore tissue perfusion, and reverse cellular ischemia. Oxygen-carrying solutions are not required until the casualty has acutely lost 30-40% of the circulating blood volume (1500-2000 ml in the average adult) [1]. Erythrocyte transfusions are safe and highly effective in restoring oxygen-carrying capabilities after severe hemorrhage.

Potential applications of erythrocyte substitutes exist in military trauma when blood is not normally available (pre-hospital settings); when blood is available, but the time required for procurement would jeopardize patient survival (emergency treatment area, unexpected massive blood loss in the operating room); and when blood is available, but the demand exceeds the supply (mass casualty situations). In this paper, we review the epidemiology of military trauma, the use of blood in field surgical facilities, and current indications for blood transfusions in trauma.

EPIDEMIOLOGY OF MILITARY TRAUMA

The incidence of wounds in battle depends on the type of military action. For example, units engaging in offensive operations sustain more casualties than units defending a position. The lethality of wounds also depends on battlefield conditions and weapons systems [2]. More gunshot and booby trap wounds occur during jungle warfare (e.g., Vietnam) than in large-scale, conventional battles (e.g., the Persian Gulf War), in which fragmentation injuries predominate.

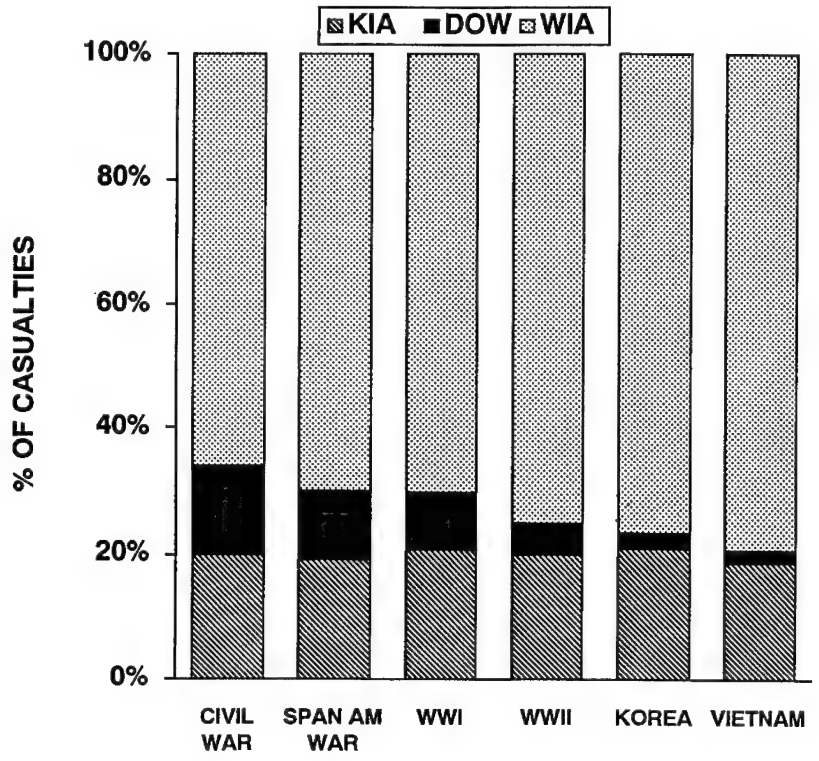

FIGURE 1. Distribution of casualties in American wars. The decrease in casualties dying of wounds (DOW) correlates with shorter evacuation times to field hospitals.

Approximately 20% of casualties are killed in action (KIA). They die on the battlefield before reaching a field hospital. This category corresponds to dead on arrival (DOA) in civilian trauma. The proportion of casualties KIA has remained unchanged throughout all the wars Americans have fought since the Civil War. In contrast, the percentage of casualties who reach a hospital, but then die of wounds (DOW) has decreased steadily (Figure 1) [3-5].

Over 50% of casualties who are killed in action bleed to death. Although massive truncal hemorrhage accounts for most of these deaths, one

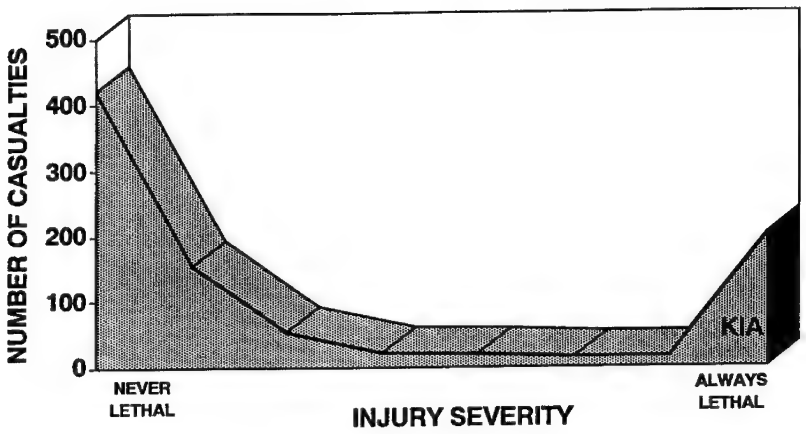

FIGURE 2. Bimodal distribution of injury severity from wounds sustained in combat. Injuries identified as *never lethal* are "carded for record only" and not admitted to a hospital. Injuries identified as *always lethal* are killed in action. Earlier treatment and evacuation could potentially reduce the *always lethal* peak.

analysis indicates as many as 22% of injuries were in regions in which hemorrhage could have been controlled by rapid application of first aid [2].

Decreasing the time required to evacuate wounded combatants from the battlefield should also affect the percentage of casualties who die on the battlefield [7], however, a greater proportion of casualties will likely die of wounds in field hospitals. Although overall hospital mortality has decreased with more rapid evacuation, those casualties who die usually do so shortly after arriving at the hospital. Hemorrhage and neurological injuries account for the overwhelming majority of hospital deaths, whereas the incidence of lethal sepsis and pulmonary failure has declined [7-9].

The severity of wounds sustained in combat follows a bimodal distribution (Figure 2) [3]. Most casualties are either killed outright or they sustain survivable, frequently minor wounds. Those who are hospitalized generally require emergency surgery to control hemorrhage or to manage soft

tissue injuries, gastrointestinal injuries or brain injuries. In Vietnam, 45-92% of casualties were operated on shortly after admission [7-9].

BLOOD TRANSFUSIONS IN MILITARY TRAUMA

Blood transfusions and intravenous fluid infusions have been shown to be lifesaving in combat casualties since World War I. Military doctrine has been developed to provide large quantities of blood to field hospitals, and a well organized logistics network (U.S. Armed Forces Blood Program) has been established to accomplish this task.

Blood is used to resuscitate casualties in severe shock, to replace blood perioperatively and to correct subsequent anemia in casualties with deficits in red cell mass after fluid replacement with crystalloid solutions. Most casualties who receive blood transfusions have hemorrhage requiring surgical control.

In Vietnam, 46% of all casualties admitted to field hospitals received blood transfusions [10]. Similar percentages were reported in the Falkland Islands campaign (64%) [11], and during the civil war in Lebanon (50%) [12]. In a recent report from the International Committee of the Red Cross (ICRC) only 16% of casualties treated in ICRC field hospitals in Thailand, Pakistan, and Afghanistan were transfused, however, almost 70% of the casualties in this series arrived at hospitals more than six hours after being wounded [13].

The number of units of blood transfused per hospitalized casualty has been reported as 1.5-2 units during World War II, Korea, and Vietnam. This number is meaningless, however, because all hospitalized patients were included in the denominator, whether or not they were hospitalized for wounds. For planning purposes, a more useful figure is the number of units transfused per casualty receiving blood. In Vietnam, patients were transfused with an average of 4.3 units [10]. Similar values were reported from Lebanon (4.6 units/casualty) [12]; however, only 2.9 units/casualty were administered by the ICRC field hospitals [13].

The amount of blood administered in field hospitals has depended on individual clinical practices and on the nature of the wounds. In general, very

few casualties have received single unit transfusions in any study, which reflects the use of blood in resuscitating severely hemorrhaging casualties before bleeding could be surgically controlled. In one series of 1,963 casualties in Vietnam, no patient received a single unit transfusion [7]. Injuries caused by land mines required more blood than those caused by gunshot wounds or fragments from bombs and rockets.

Universal donor (Type O, Rh positive) blood was widely used before 1966; most blood used subsequently has been fully cross-matched. The administration of universal donor blood is extremely safe; more than 100,000 units were given in Vietnam without a single fatal hemolytic transfusion reaction. Typing and cross-matching, however, can be performed within 20 minutes [14]. For casualties requiring multiple units, administering low titer, type O or type-specific blood prevents the risk of antibody transfer that can occur from the anti-A and anti-B antibodies present in normal titer, type O blood.

INDICATIONS FOR TRANSFUSION

Clinical criteria for erythrocyte transfusions are not well delineated. The awareness of infectious risks and a better understanding of oxygen delivery has led to much more judicious use of blood in 1993 than was practiced 40 years ago. Anecdotal descriptions of blood transfusions in the Korean War report casualties being transfused to hematocrits of around 40% [15]. Pre- and post-transfusion hematocrits have not been reported for individual casualties, nor have specific "transfusion triggers" for battlefield casualties been well established.

NATO doctrine for wartime blood transfusion follows guidelines established by the American College of Surgeons for the Advanced Trauma Life Support (ATLS) course [16-17]. Blood transfusion is indicated for casualties with evidence of ongoing hemorrhage in the presence of shock and for those casualties whose vital signs either fail to respond or respond only transiently to

volume infusion. Guidelines have also recommended administering blood to casualties bleeding more than 100 ml/min.

Clearly, more objective criteria are required. The first therapeutic objective in bleeding patients is to control hemorrhage. Controlling hemorrhage is the primary modality for preventing the consequences of blood loss and will immediately reduce subsequent transfusion requirements. Although this fact would seem intuitively obvious, as many as 20% of all casualties who died on the battlefield in Vietnam could have been saved by simple first aid measures to stop bleeding [2]. In some casualties, however, blood loss cannot be controlled without surgery. It is these casualties who require aggressive resuscitation to prevent death.

In patients with ongoing hemorrhage or severe blood loss, the most important immediate objective is to ensure adequate perfusion of cells and tissues. There is usually abundant reserve in the body's oxygen delivery system in young people, thus volume replacement with oxygen-carrying solutions is generally not required in the initial phase of resuscitation. Restoring intravascular volume with crystalloid or colloid solutions increases perfusion and restores oxygen delivery to peripheral tissues. Even in the absence of oxygen delivery, maintained perfusion prevents cell death by removing toxic metabolites and by delivering substrates for anaerobic metabolism to tissues.

The minimally acceptable hemoglobin concentration is an individual characteristic that depends on non-hemoglobin variables, including the ability to increase cardiac output, tissue oxygen demand, pH, the ability to oxygenate available hemoglobin, and the adequacy of perfusion to critical vascular beds [18]. An oxygen extraction ratio of greater than 50% [19] or a mixed venous oxygen saturation of less than 67% (mixed venous oxygen content = 35 mm Hg) [20] have been suggested as critical levels for transfusion.

Because these values are not readily available in most emergency situations, hemoglobin concentrations have been used to identify casualties requiring blood transfusion. As hemoglobin concentrations of less than 10 g/dl result in decreased oxygen delivery to the myocardium, this value has been identified as an indicator for erythrocyte transfusion [21]. In fact, data derived from older patients with preexisting cardiovascular disease do not necessarily

correlate with adverse outcomes in young trauma patients with abundant cardiac reserve. Animal studies have shown that hemoglobin concentrations of 5 g/dl after exchange transfusion are well tolerated [22]. More recent recommendations suggest hemoglobin concentrations below 7 g/dl [17] to 8 g/dl [12] as "triggers" for transfusion. In trauma patients, hemoglobin concentrations or hematocrits may not accurately reflect the intravascular volume status in the acutely hemorrhaging trauma patient. In the Danang Naval blood utilization study, the mean admission hematocrit was 36.5 ± 5.3% [23].

Hemoglobin concentrations that are well tolerated in anemic volunteers or animals who are otherwise healthy may not be safe for military trauma casualties. Additional encroachments on tissue oxygen supply may result from increased cellular metabolic requirements, arterial hypoxemia, or alkalosis, leaving little physiologic reserve at lower hemoglobin concentrations.. Also, the potential for close medical monitoring for ongoing hemorrhage is limited in the austere settings of field hospitals, and the availability of other supportive measures that could increase tissue oxygen delivery, such as ventilators, is limited [20].

PLANNING FOR MILITARY BLOOD USE

At the height of the Cold War, plans for military blood use were based on scenarios of high-intensity combat involving field armies with millions of soldiers on the plains of northern Europe. Blood requirements of more than 100,000 units a day were predicted. At that rate, blood use would have been more than three times the sustained yield of the entire U.S. blood banking system. Additional logistic constraints requiring that blood be available on short notice and that it not monopolize limited airlift capabilities became the driving forces in the development of alternatives to liquid blood. As the Soviet military threat has diminished, U.S. military blood requirements have been markedly reduced.

Estimates of blood use based on previous U.S. military experience suggest far more modest blood requirements. Even the largest demands for

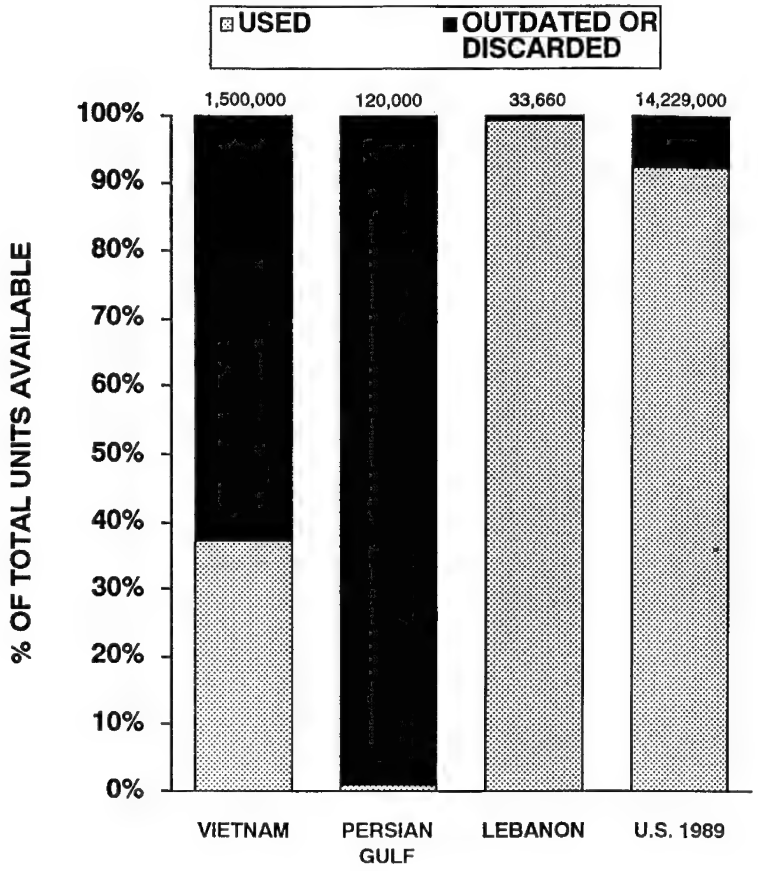

FIGURE 3. Patterns of blood use in recent conflicts, compared to the total U.S. blood supply in 1989 [26]. The high percentage of units outdated or discarded in the Vietnam and Persian Gulf Wars reflects the need to position adequate blood to meet anticipated needs far from the continental U.S.

blood faced in 1968, when 476,000 units were shipped to Vietnam [24], or in 1990, when 120,000 units were shipped to the Persian Gulf [25], represent less than 4% or 1%, respectively, of the annual blood supply in the United States (Figure 3). Although 60% of the blood shipped to Vietnam and 95% of the blood sent to the Persian Gulf became outdated, the costs to provide guaranteed availability are willingly borne by military planners.

POTENTIAL ROLES FOR ERYTHROCYTE SUBSTITUTES IN MILITARY TRAUMA

Potential military uses of erythrocyte substitutes are to replace blood for transfusion therapy and to extend the availability of oxygen-carrying solutions to applications for which blood is not currently available. To replace blood, erythrocyte substitutes must compare favorably to blood in terms of safety, efficacy, durability, and cost effectiveness. When used to extend the availability of oxygen-carrying solutions, erythrocyte substitutes must meet the same criteria, as well as show a therapeutic advantage compared with standard crystalloid resuscitation solutions.

The safety of both universal-donor and type-specific blood is well documented, as is erythrocyte viability and survival in banked blood. The efficacy of erythrocyte transfusions in managing hemorrhagic shock has been well substantiated for over half a century. Therefore, for erythrocyte substitutes to be competitive as a blood replacement, there must be significant advantages in lower cost, less volume and weight per unit dose, and less stringent storage requirements.

Extending the role of oxygen carrying solutions with erythrocyte substitutes to pre-hospital resuscitation of hemorrhagic shock will also require documentation of the advantages of an oxygen-carrying solution over crystalloid solutions in casualty survival rates and morbidity. In scenarios in which evacuation times to definitive care are rapid, justifying a therapeutic advantage of erythrocyte substitutes will be difficult unless they are very safe. Erythrocyte substitutes could be beneficial to medical units supporting airborne and special operations units with limited capabilities for providing definitive care to large numbers of casualties.

The opinions and assertations contained herein are the private views of the authors and are not to be construed as official nor do they reflect the views of the Department of the Army or the Department of Defense (AR360-5).

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ART. CELLS, BLOOD SUBS., AND IMMOB. BIOTECH., 22(2), 159-169 (1994)

ASSESSMENT OF BLOOD SUBSTITUTES: I. EFFICACY STUDIES IN ANESTHETIZED AND CONSCIOUS RATS WITH LOSS OF 1/3, 1/2 AND 2/3 BLOOD VOLUME

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ABSTRACT:

Acute and long-term recovery (14 days) studies were conducted in conscious rats bled 1/3, 1/2, and 2/3 blood volume, and in anesthetized rats bled 1/3 volume. Study I : bled 1/3 blood volume. Study Ia : Anesthetized rats bled 1/3 blood volume were in shock and most died within 1 hour; the group which received infusion of 3x volume Ringer's lactate regained blood pressure with 100% long-term recovery. Study Ib: In conscious rats bled 1/3 blood volume, blood pressure did not fall to shock levels, and the long-term recovery in both the control group and the group which received 3x volume Ringer's lactate was 100%. Study II: Bled 1/2 blood volume. In conscious rats bled 1/2 volume, the blood pressure of the control group was slightly above 70mm Hg. Infusion of 3x volume Ringer's lactate or 7 gm% human albumin in Ringer's lactate increased blood pressure to above 90mmHg in the acute study. Long-term recovery and survival rate was 80% in both the controls and the Ringer's lactate group, and 100% in the albumin group. Study III: bled 2/3 blood volume. In conscious animals, loss of 2/3 blood volume resulted in fall of blood pressure to shock levels and death of the animal within 1 hour. Infusion of 3x volume Ringer's lactate did not increase blood pressure to above shock level. Stroma-free hemoglobin and polyhemoglobin increased blood pressure, and whole blood returned blood pressure to normal. In long-term recovery, poly-hemoglobin, but not SFHb, was as effective as whole blood with 100% long term recovery. Animal models in Study I and II are suitable only for studies of volume replacement. Animal models in Study III, especially using long-term recovery studies, are more suitable for studying both volume and red blood cells replacements..

INTRODUCTION:

Blood loss which is severe enough to require replacement usually occurs in patients whose conditions are also complicated by trauma, dehydration, diseases, general anesthesia, or any combination thereof. Studies using models with these complications are valuable, but they require multifactorial analysis. We feel that it is important to start with conscious normal animals in assessing the efficacy of replacing lost blood so that there is only one variable to be studied. Once sufficient basic results have been obtained from this basic model, further research with more complicated models can be justified.

Before the advent of blood substitutes, most of the studies on replacement of blood loss were based on volume replacement [1]. Most animal models are therefore based on studying the efficacy of volume replacement rather than red blood cell replacement. The various studies have investigated different volumes of blood loss using different types of animal models. Some investigations have been based on acute studies with a few hours of followup, and others have studied long-term recovery. These different approaches have not been compared using the same animal model in the same laboratory. In the present study we used a conscious animal model to compare the replacement of a blood loss of 1/3 volume (23.33 ml/kg); 1/2 volume (35 ml/kg) and 2/3 volume (46.66 ml/kg). We also studied the effects of general anesthesia in rats that lost 1/3 blood volume.

METHODS:

Chronic cannulation of femoral artery and vein:

Using methods reported earlier by Tabata and Chang [2], we cannulated the femoral artery and vein by inserting catheters and exteriorizing them through the tail vein. The catheters were protected by a removable shaft which covered this section of the tail. After chronic cannulation, the rats were allowed to move about freely with no restraints. During the experiment, the shafts were removed and the catheter connected to monitor blood pressure or to remove or replace blood. The animals remained conscious at all times, similar to patients on hemodialysis.

Preparation of animal for conscious rat experiments:

The animals were stabilized for a minimum of 7 days, then chronically cannulated as described. Hematocrits were measured to ensure that they were within the normal range. The animals were allowed to recover in the animal centre with free access to a normal diet. After 7 days, hematocrits and body weights were measured. Only animals with no decrease in hematocrit or body weight were used in the experiments. The success of chronic cannulation depends on the experience of the person who carries out this rather complicated proceedure. As the experience of the person increases, the proportion of animals showing no change in hematocrit or body weight during the 7 days reovery likewise increases. Any decrease in hematocrit or body weight indicates chronic blood loss, dehydration, or other problems. All rats showing no changes were randomly assigned to the following studies.

Hemorrhagic shock induction and replacement

This procedure has been described in detail in our previous publications [3,4]. We used Sprague-Dawley rats, 340± 40 gm, purchased from Charles River Laboratories (Wilmington, MA). Blood was removed at 0.5 ml/min. During the removal of 1/2 and 2/3 blood volume, we paused for 10 min after 36% of the volume had been bled, then we resumed the bleeding, similar to the Wigger's model [5]. Blood was replaced with shed blood, polyhemoglobin, stroma-free hemoglobin or other solutions at 0.5 ml/min.

Acute studies:

Blood pressure, respiration, and heart rate were monitored continuously during the control period, bleeding period, replacement period, and 1 hour thereafter. Hematocrit, hemoglobin, and other measurements were also taken.

Long-term recovery:

After the acute studies, the animals were placed in cages and returned to the animal center. The shaft and external part of the cannulae were removed after 3 days. The rats were given free access to a normal rat diet and water, and monitored for 14 days. Hematocrit, body weights , general well-beings etc were followed. Those that survived

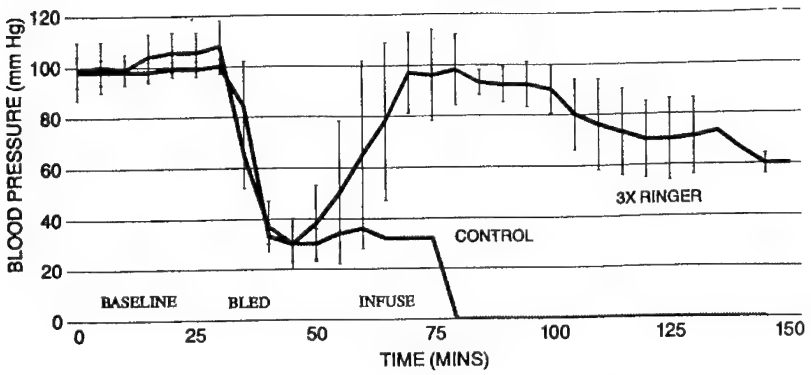

FIGURE 1: Anesthetized rats with loss of 1/3 blood volume (23.33 ml/kg). (Expressed as mean and standard deviations)

for 14 days were considered to have recovered completely. Since in previous studies, all rats which have survived for 14 days continued to survive.

RESULTS AND DISCUSSION:

Anesthetized or conscious rats with loss of 1/3 blood volume (23.33 ml/kg)

1. In anesthetized rats, a blood loss of 23.33 ml/kg resulted in a rapid decrease in blood pressure to <40 mm Hg (FIGURE 1). Infusion of Ringer's lactate at three times the shed blood volume (3x volume) rapidly increased blood pressure to normal. Blood pressure remained above shock level throughout the observation period. In the control animals not receiving fluid replacement, blood pressure remained at shock level, and most died within 1 hour.

2. Another group of rats under lighter grades of anesthesia (corneal reflex present) was also studied. Decreases in blood pressure were less marked.

3. The results in conscious rats were surprisingly different from anesthetized rats. As shown in FIGURE 2, the loss of 1/3 blood volume in conscious animals resulted in only small decreases in blood pressure which did not reach shock level. Furthermore, the

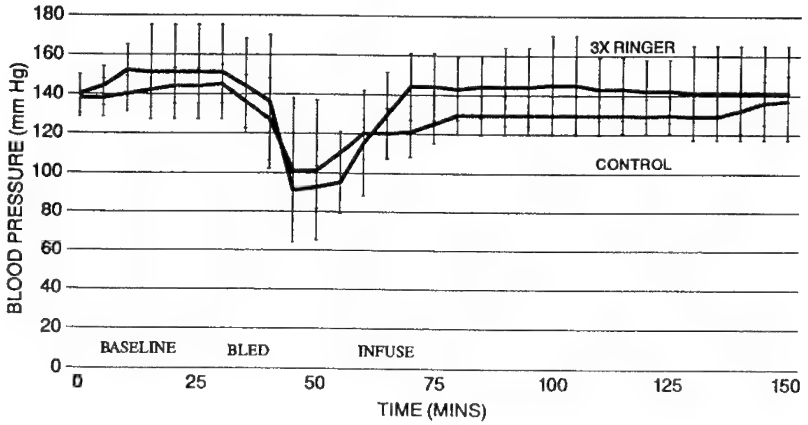

FIGURE 2: Conscious rats with loss of 1/3 blood volume (23.33 ml/kg).

recovery of blood pressure in the control group was not significantly different from that in the group which received 3x volume Ringer's lactate.

The long-term recovery rates at 14 days are shown in FIGURE 3. In the anesthetized groups, replacement with 3x volume Ringer's lactate resulted in 100% long-term recovery. In anesthetized groups without Ringer's lactate, the long-term recovery was significantly less and depended upon the degree of anesthesia. In the conscious rats, both those receiging 3x volume Ringer's lactate and those with no infusion had survival rates of 100% at day 14 (FIGURE 3).

These results show the marked differences in response between conscious and anesthetized animal models. This further point out the importance of defining the exact animal model for the specific application in studying blood replacement. This study shows that volume replacement alone using 3x volume Ringer's lactate is extremely important for survival in 1/3 volume blood loss in anesthetized animals. On the other hand, conscious animals with the same degree of blood loss did not require volume replacement.

Conscious rats with loss of 1/2 blood volume (35 ml/kg)

After conscious rats had been bled 35 ml/kg, blood pressure was measured at shock level, about 60 mm Hg (FIGURE 4). In the control group, blood pressure remained at

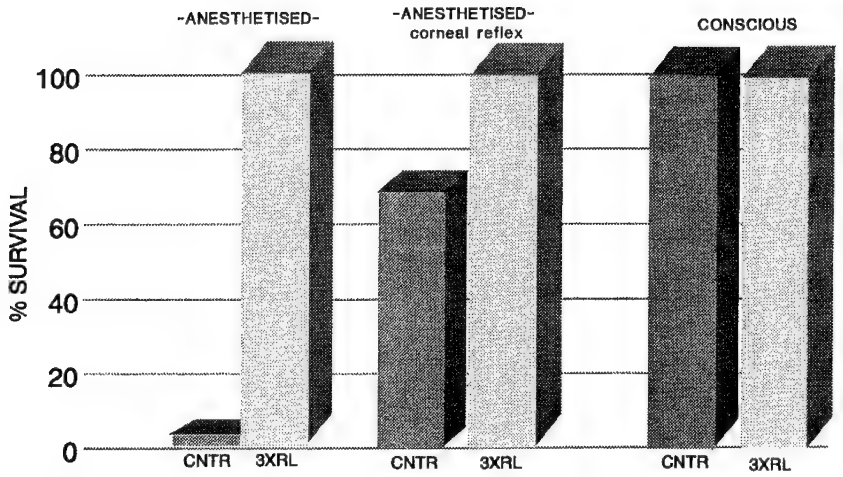

FIGURE 3: Long term recovery rates of anesthetized and conscious rats with loss of 1/3 blood volume (23.33 ml/kg).

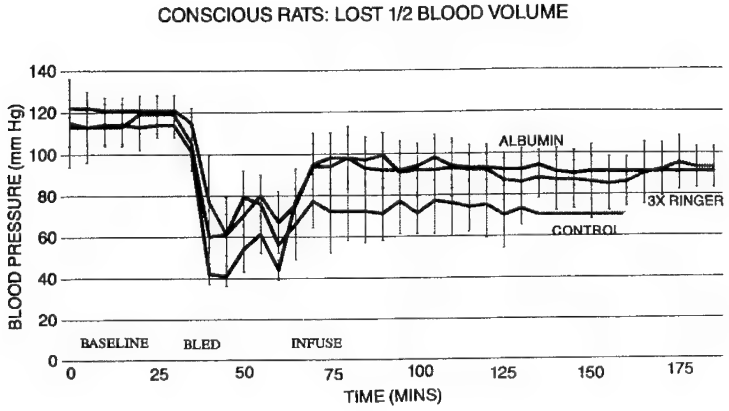

FIGURE 4: Conscious rats with loss of 1/2 blood volume (35 ml/kg).

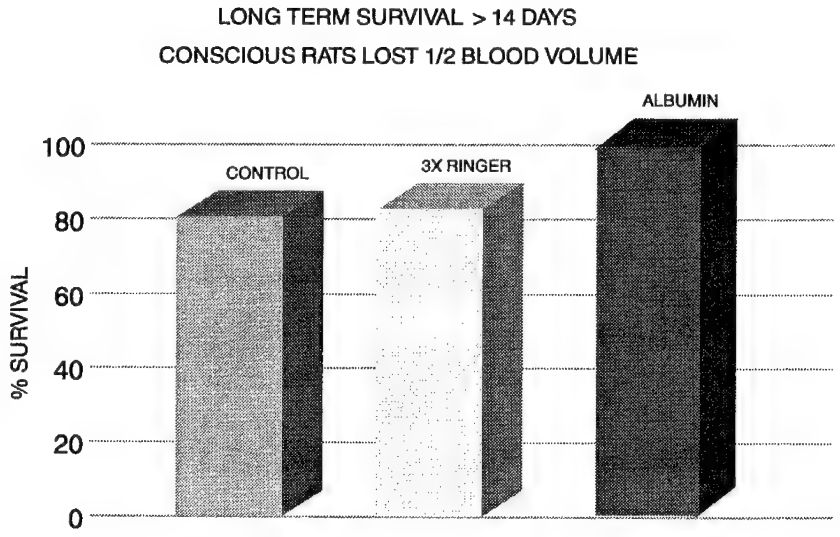

FIGURE 5: Long-term recovery rates of conscious rats with loss of 1/2 blood volume (35 ml/kg).

shock level. In the group that received 3x volume Ringer's lactate, the blood pressure increased to above shock level, and was maintained throughout the acute period of study. In the group which received 1x volume replacement with 7 gm% human albumin in Ringer's lactate solution the blood pressure also increased and reamined at above shock level (FIGURE 4).

Long-term recovery and survival rate at 14 days for rats bled 1/2 volume was 80% in both the control group and in the group receiving 3x volume Ringer's lactate (FIGURE 5). However, in the group which received 1 volume albumin-Ringer's lactate, the survival rate was 100%. The albumin contributed to oncotic pressure not present in Ringer 's lactate solution. If one were to use crosslinked hemoglobin solution in the present study, without using albumin as control, one would make the erronous conclusion that crosslinked hemoglobin is effective as a red blood cell substitute. If the albumin control has been used in the present study, we can show that, the crosslinked hemologbin, like albumin may just be acting as effective oncotic pressure agents. Indeed, it would appear

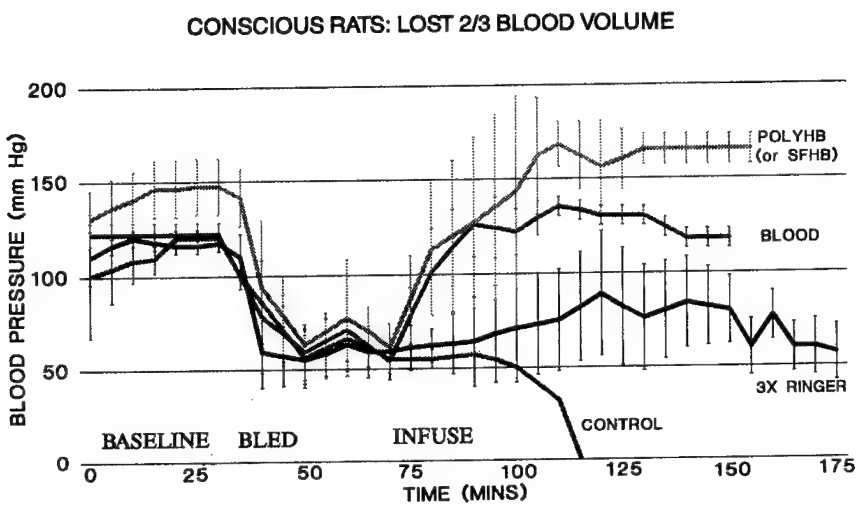

FIGURE 6: Conscious rats with loss of 2/3 blood volume (46.66 ml/kg).

from the present study, that in 1/2 volume bled conscious animals, there is only need for volume replacement with oncotic agent, there is no need for red blood cell replacement.

Conscious rats with 2/3 blood volume loss (46.66 ml/kg)

Removal of 2/3 blood volume in the control group resulted in a rapid decrease in blood pressure to shock level with the rats dying within 1 hour (FIGURE 6). In rats receiving 3x volume Ringer's lactate, blood pressure did not recover significantly and remained at shock level. In animals receiving whole blood, blood pressure returned to normal. Blood pressure increased in animals that received stroma-free hemoglobin or polyhemoglobin. In the acute study, stroma-free hemoglobin, polyhemoglobin and whole blood were equally effective (FIGURE 6).

However, results in the long-term recovery study were completely different (FIGURE 7). In long-term recovery from 2/3 volume blood loss, poly-hemoglobin was as effective as whole blood with 100% survival. However, survival rates was 0% in the control group, less than 40% for stroma-free hemoglobin; less than 50% for 1x volume albumin in Ringer's Lactate and less than 20% for 3x volume Ringer's lactate. Follow-ups also

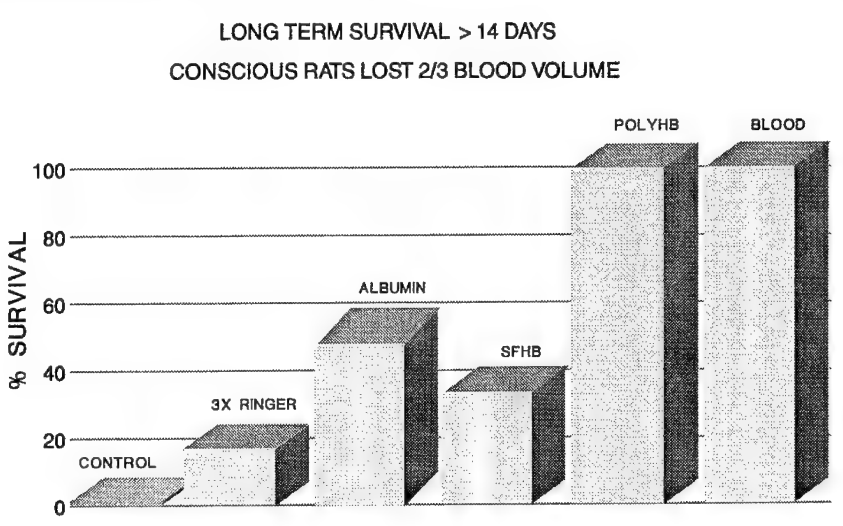

FIGURE 7: Long term recovery rates of conscious rats with loss of 2/3 blood volume (46.66 ml/kg).(from reference 3).

include body weight, general well beings and other factors. Body weight gains in those groups which received whole blood or polyhemoglobin were similar. The survival rates results point out the importance of conducting long-term recovery studies rather than just short-term acute studies.

GENERAL DISCUSSION

A blood volume loss of at least two-thirds was required in the conscious normal rat for adequate assessment of the efficacy of fluid and red blood cell replacement. With a two-thirds loss of blood volume, polyhemoglobin was as effective as whole blood in the long-term recovery. Plasma expander was less effective than polyhemoglobin or whole blood, but slightly more effective than 3x volume Ringer's lactate which was slightly more effective than no treatment. These graded responses permitted differentiation of the effects between 1. control. 2. volume replacement alone and 3. volume replacement combined with red blood cell replacement. Polyhemoglobin is as effective as whole blood

in the present model. On the other hand, if we want to go one step further to compare the efficacy of polyhemoglobin and whole blood, the two-thirds blood volume loss conscious model may not have been severe enough.

With the efficacy now demonstrated for polyhemoglobin to replace red blood cells in conscious normal animals, analysis can proceed for more complicated situations, such as the effects of blood volume replacement in patients with trauma, dehydration, disease, general anesthesia, or a combination thereof [6-9]. However, it should be noted that these evaluations may be extremely complicated. For example, although 3x volume Ringer's lactate replacement for hemorrhagic shock due to trauma is in routine clinical use, it has not been possible to demonstrate whether it is effective in those with uncontrolled hemorrhage [10].

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ART. CELLS, BLOOD SUBS., AND IMMOB. BIOTECH., 22(2), 171-180 (1994)

ASSESSMENT OF BLOOD SUBSTITUTES: II. IN-VITRO COMPLEMENT ACTIVATION OF HUMAN PLASMA AND BLOOD FOR SAFETY STUDIES IN RESEARCH, DEVELOPMENT, INDUSTRIAL PRODUCTION AND PRECLINICAL ANALYSIS.

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ABSTRACTS

Animal safety study cannot predict the effects of blood substitutes in human response . Response of human, especially in immunology and complement activation, need not be the same as those in animals. We have earlier reported an in-vitro preclinical screening test based on testing the effects of modified hemoglobin on complement activation of human plasma or blood in vitro .

In this test, modified hemoglobin is added to human plasma in a test tube. Complement activation is followed by the C3a levels. Since this directly measures the effect of modified hemoglobin on human plasma, it would be the closest response in human next to injecting this into human. Thus, this could be an important bridge before clinical use in patients. However, why wait for the completion of research, industrial production and preclinical animal studies? Why don't we do this test right at the beginning during the research stage? If a new system is found to cause complement activation at this stage, one can avoid tremendous waste of time and money in further development, industrial production and preclinical animal study. This paper analyzes this appraoch in research, development, industrial production and preclinical analysis.

INTRODUCTION

Aims of present research

Research and development on blood substitute involve the stages of (1) research, (2) industrial production, (3) preclinical animal studies, (4) clinical trials. The most critical problem at present is that animal safety study cannot predict the effects of blood substitutes in human response (1). Response of human, especially in immunology and complement activation, need not be the same as those in animals. We have earlier reported an in-vitro preclinical screening test based on in-vitro complement activation of human plasma (2-4). In this test, modified hemoglobin is added to human plasma in a test tube. Complement activation is followed by the C3a levels. Since this directly measures the effect of modified hemoglobin on human plasma, this could be an important bridge before clinical use in patients.

However, why wait for the completion of research, industrial production and preclinical animal studies? Why don't we do this test right at the beginning during the research stage? If a new system is found to cause complement activation at this stage, one can avoid tremendous waste of time and money in further development, industrial production and preclinical animal study. This paper analyzes this appraoch in research, development, industrial production and preclinical analysis.

Complement Activation:

Complement activation is important in a number of potential adverse reactions of human to modified hemoglobin (5-7). Modified hemoglobin may contain trace amount of blood group antigen which can form antigen-antibody complex. This can be detected by complement activation. Other potential materials can also cause complement activation. These include endotoxin, microorganism, insoluble immune-complexes, chemicals, polymers, organic solvents and others.

METHODS

In vitro screening test based on human plasma

The exact details are very important and can be found in our earlier publications (2-4). It is summarized very briefly here (Fig. 1). In the routine measurement of C3a level in human, blood is removed and EDTA added. The plasma is then separated and analyzed for C3a. However, the in-vitro preclinical screening test involves much more complicated procedures (Fig. 1). Blood is collected in heparin. Heparin is used instead of EDTA because EDTA inhibits

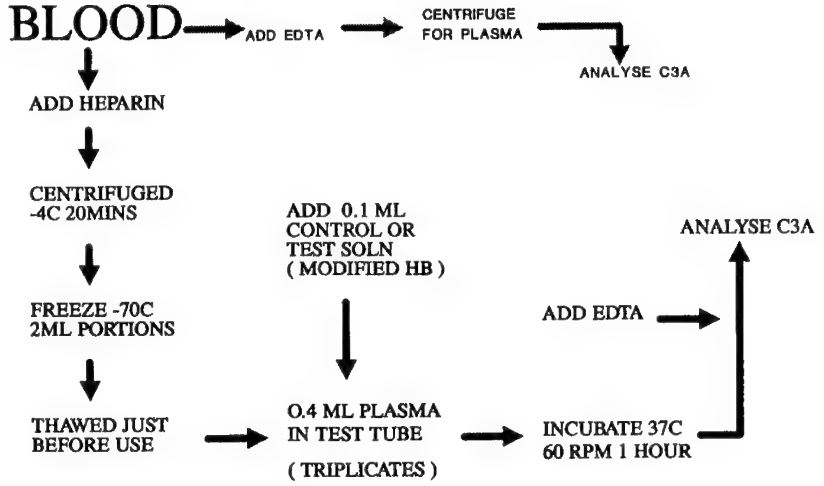

Figure 1: Schematic representation of in-vitro screening test for blood substitutes. Top line represents procedure for measurement of C3a level in plasma. Bottom schedule represents in vitro screening test by adding modified hemoglobin to human plasma in test tubes.

complement activation. The heparinised blood in special tubing is then centrifuged at 4°C immediately. The plasma obtained is frozen in small aliquot at -70°C. The required aliquots are removed and thawed just before use. 0.4 ml of plasma are used in each test. 0.1 ml of control or modified hemoglobin is added to each 0.4 ml of plasma. These are incubated at 37°C at 60 rpm for 1 hour. EDTA is added to stop complement activation and the plasma is immediately analyzed for C3a. C3a measurement is based on an immunoassay assay (Amersham, Canada).

RESULTS AND DISCUSSIONS

I. Use in research : example in screening of materials used in nanocapsule preparation

In research on blood substitute, different chemicals, reagents and organic solvents are used. This includes crosslinkers, lipids, solvents, chemicals, polymers and other materials. Some of these can potentially result in complement activation and other reactions in humans. Other potential sources of problems include trace contaminants from ultrafilters, dialysers, chromatography.

In our ongoing study of hemoglobin nanocapsules (8,9), different polymers, lipids, reagents and solvents are used. We therefore analyzed their effects on complement activation of human

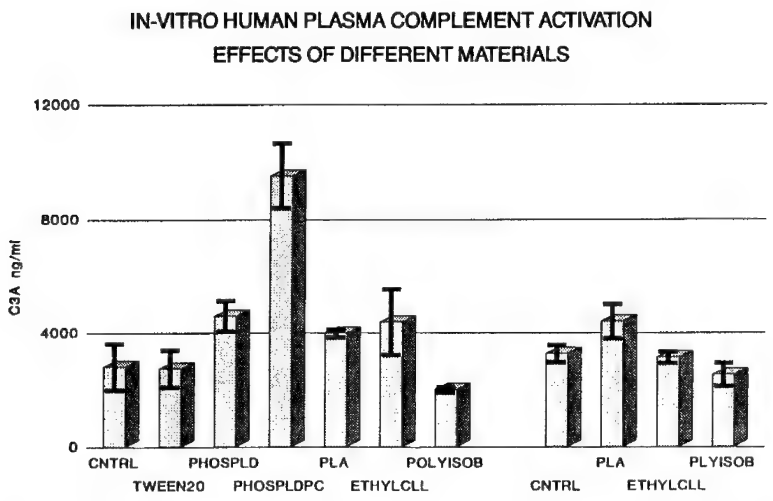

Figure 2. Effects of different materials on in-vitro human plasma complement activation The group on right - after repeated aqueous washing.

plasma in-vitro. The results are shown in Figure 2. One type of L- -phosphatidylcholine caused very marked increase of complement activation. Another type of L- -phosphatidylcholine did not result in marked increase in complement activation. Polymers tested like polylactic acids did not result in much complement activation. After repeated washing, there was no longer any significant complement activation (Fig. 3). Another polymer, isobutyl 2-cyanoacrylate, resulted in less C3a level than the control. The reason for this is that the polymer does not cause complement activation, at the same time, it also adsorbs C3a. The emulsifying agent Tween 20 also did not result in complement activation.

In-vitro screening of organic solvents:

The effect of different types of organic solvents was also studied. Ten microliters were added to 400 microliters of plasma. The effect of complement activation is shown in Figure 3. At this concentration, ethanol, acetone and ethylene acetate result in marked complement activation. The other organic solvents did not cause this degree of complement activation. This is important because sometimes polymers and lipids are dissolved in solvents. If these polymers or lipids dissolved in organic solvents are tested for complement activation, the organic solvent itself would cause complement activation. Thus, in testing polymer or lipid it is important to be sure that they are free from organic solvents.

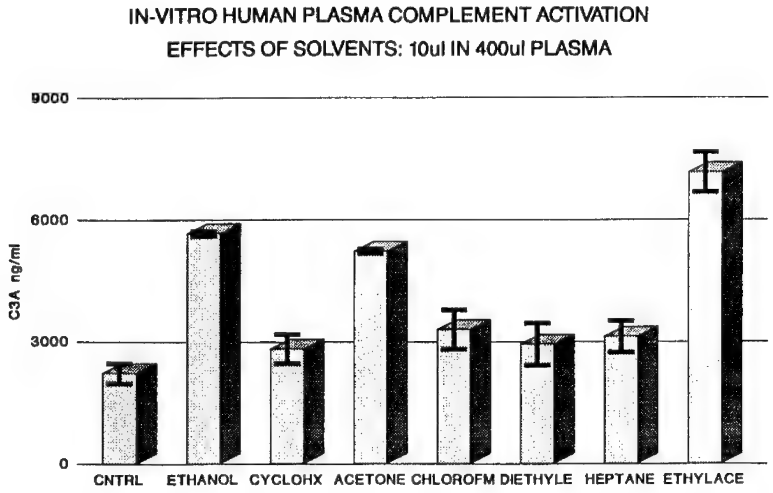

Figure 3: Effects of solvents on in-vitro human plasma complement activation

In the final nanocapsules blood substitute or other types of blood substitutes, most of the organic solvents would be removed. Thus, the next step is to see the effect of trace amounts of organic solvents on complement activation. In this study, the organic solvent which causes the highest complement activation, ethyl acetate was used. The results in Figure 4 show that ethyl acetate at 1/400 dilution no longer causes complement activation.

III. Correlation of in-vitro complement activation to clinical symptoms

What are the clinical implications of C3a levels in the above in-vitro complement activation screening test? Until actual clinical data is available, one cannot conclusively establish this. On the other hand, there are extensive clinical data in the use of different types of hemodialysis membranes and their relationships to complement activation and anaphylactic reactions (10). In patient using dialysis membrane which did not cause complement activation, the postdialysis C3a levels in the plasma were less than 1000 ng/ml (Fig. 5). In patients using membranes which caused complement activation, the C3a levels were significantly increased. There was no clinical symptoms if the levels were less than 3000 ng/ml. Clinical symptoms appeared when C3a was above this level. Increasing levels resulted in increasing severity in the symptoms. Symptoms include: myalgia, chest tightenss, fever, chill and others. These are similar to some of the observed adverse reactions in earlier phase 1 clinical safety studies in human from some centres.

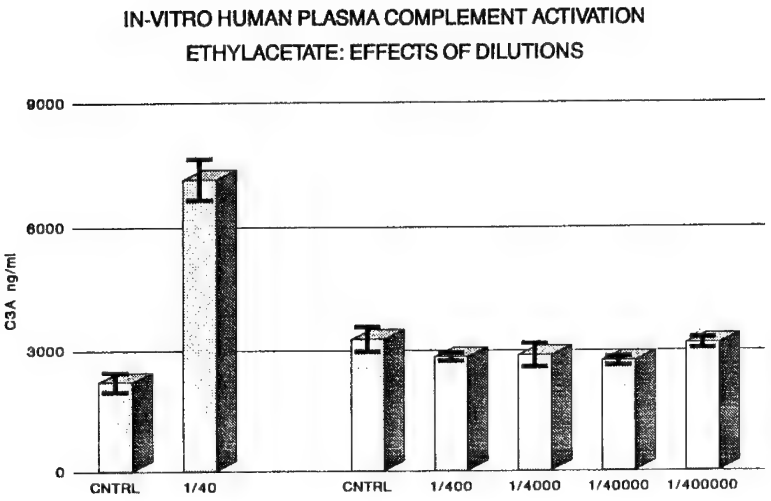

Figure 4: Effects of dilution of ethylacetate on in-vitro human plasma complement activation.

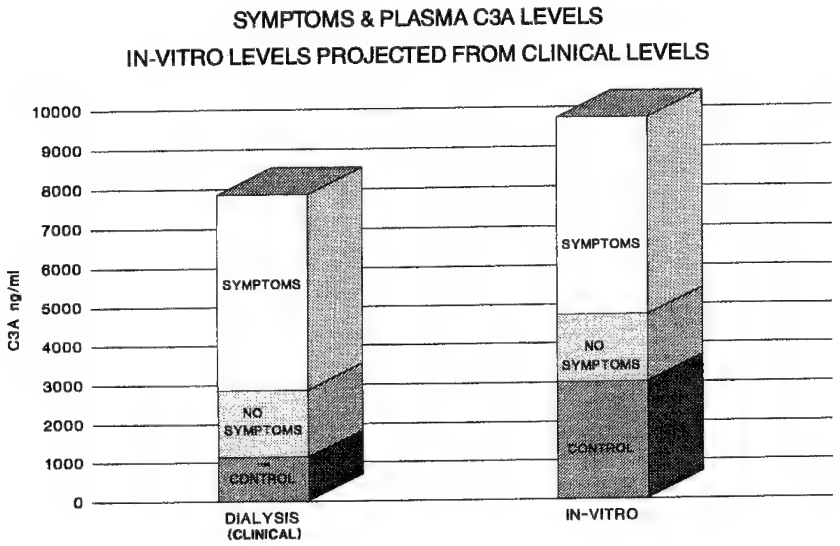

Figure 5: Projection of clinical results in dialysis patients to in-vitro screening test.

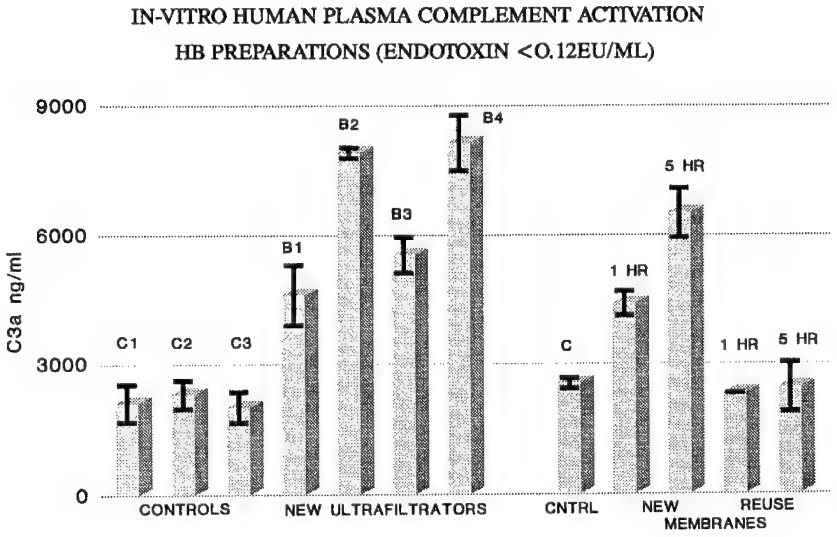

Figure 6: Sucessful use of in-vitro test in industrial scale-up production of polyhemoglobin. The use of new ultrafiltration membrane can result in hemoglobin preparations which can cause complement activation when tested in-vitro in human plasma. In-vitro screening test has therefore eliminated this potential problem right at the beginning (From ref 11)

The following three factors are important in projecting these to the in-vitro screen test. Extra manipulations and storage of blood and plasma (Fig. 1) in the in-vitro screening test resulted in increased control C3a levels compared to those obtained immediately from patients. The control C3a levels in the in vitro tests are therefore between 2000 and 3000 ng/ml. Therefore, in projecting to clinical levels, one has to add the additional control levels to these control levels (FIG 5). Triplicate control samples must be used in each batch of analysis.

The volumes used in the in-vitro test were 100 microliters of modified hemoglobin in 400 microliters of human plasma. If we consider the plasma volume in an average man, this would be equivalent to about 500 ml of blood substitute to the total plasma volume. This is comparable to the amount used in phase 1 clinical trial in humans. Of course, higher ratios can also be used.

C3a is a smaller molecule (M.W. 9,000) than C3 (M.W. 180,000). Therefore once it is formed, C3a equilibrates rapidly across the capillaries. In measuring C3a in dialysis patients, one has to do this within very short intervals to catch the peak rise in C3a. The peak is reached in

the first 15 minutes. After this, it declines rapidly to normal in 60 mins. In the in-vitro study, the C3a would not escape from the test tube, therefore, the maximal level of C3a would be available.

III. Use of in-vitro screening test in industrial production

As described elsewhere (11), we have also used this preclinical test to help others in industrial production . Thus in the industrial scale-up of polyhemoglobin, this in-vitro screening test showed that certain batches caused complement activation. By using this test further, it shows that this is the result of the use of new ultrafiltrators. Re-used ultrafiltrators did not cause complement activation (Fig. 6). This is an example of the importance of using this in-vitro test in industrial production. Without this test, some batches could result in adverse effects of "unknown causes" in human. Chromatography, ultrafiltrators, dialysis membranes and other separation systems are used extensively in the preparation of different types of blood substitutes. It is therefore important to screen for the possibility of trace contaminants that could cause complement activation. In the same way, different chemical agents and different reactants used in industrial production could be similarly tested.

IV. Clinical trials and use in human:

This in-vitro test may be useful in large scale screening for human response. For instance, it could be used to study variation in production batches. It could also be used to study individual variations. Furthermore, it could also be used to analyze the response of different human population, especially with different disease conditions. It is important to note that all these could be done without ever introducing any blood substitute into the human.

V. Use of blood instead of plasma for in-vitro test

In these large scale clinical studies described in the above paragraph, the use of plasma requires obtaining blood and then centrifuging to separate the plasma. For use in research and industrial screening, large samples of human plasma can be carefully prepared and stored at -70C for use. However, to screen large populations., we may want to eliminate the extra step of centrifugation. Our recent studies show that it is possible to use small sample of heparinized whole blood obtained directly from finger pricks and use immediately for analyzing complement activation (12). This in-vitro test using human blood instead of human plasma may be most useful in large scale screening of a large number of patients. On the other hand, for laboratory research and industrial scale-ups and productions, it may be more convenient to use plasma

samples. Since plasma can be prepared and stored frozen at -70C and used at anytime. Examples of these situations include as described above.

Acknowledgement

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MECHANISMS AND EFFICACY OF FLUOROCHEMICAL OXYGEN TRANSPORT AND DELIVERY

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ABSTRACT

Fluorochemicals for medical use are metabolically inert liquids with a high solubility for gases, and can dissolve 50 percent or more of their own volume of oxygen (O_2) at ambient pressures. The solubility is directly proportional to the oxygen tension (PO_2) and transport is thus not saturable, unlike the O_2 saturation of hemoglobin (Hb) which follows the well known "S" shaped relationship with PO_2. Intravenously-injected emulsions of fluorocarbons transport only about one seventh as much O_2 as Hb on a gram for gram basis, even when high concentrations of O_2 are respired. However, because of the high O_2 extraction from fluorocarbons in these circumstances, their contribution to O_2 consumption is more than 65% of that of Hb.

O_2 delivery to the tissues depends on the product of the cardiac output and the arterial oxygen content. When red cells are transfused, blood viscosity increases and cardiac output decreases. This lessens the efficacy of blood in comparison to that of fluorocarbons, and increases the relative transport of O_2 in the metabolically readily-accessible plasma compartment. This provides an interesting application for low dose fluorocarbons during surgery in combination with autologous blood use. The efficacy of fluorocarbons in this setting can be predicted by computer modelling.

INTRODUCTION

Fluorocarbons, or more accurately, perfluorochemicals (PFCs), are relatively simple aromatic or aliphatic compounds in which all hydrogen atoms have been replaced by fluorine atoms. The strength of the carbon fluorine chemical bond [1] and the shielding effect of the densely packed fluorine atoms makes PFCs both chemically and biologically inert [2]. One study published in the chemical literature [3] has suggested that metabolism of PFCs can occur in vivo. This assertion has been vigorously denied by chemists and biochemists working in the field of PFC emulsions for biological use. Early work by Yokoyama et al [4], which demonstrated that, although excretion rates affected the relative amounts of different PFCs present in the liver, no new gas chromatograph peaks could be detected, indicating no metabolic processing of the compounds. Additionally, no increased fluoride concentrations were seen in either animals [4] or man [5] following PFC emulsion administration.

PFCs have a high solubility for respiratory gases and, though almost completely insoluble in water, PFCs can be formulated as stable emulsions that can then be injected intravenously as oxygen-transporting blood substitutes. The oxygen (O_2) transport capacity of these PFC emulsions depends on the PFC concentration, the partial pressure of oxygen (PO_2) to which they are exposed (Henry's law applies), and the O_2 solubility of the PFC in question. Thus, highly concentrated emulsions in equilibrium with high PO_2 environments can transport more O_2 than dilute emulsions not exposed to high O_2 concentrations. Before O_2 transport and delivery by PFCs can be considered, it is first necessary to have a thorough understanding of O_2 transport in the blood.

OXYGEN TRANSPORT IN THE BLOOD

O_2 is normally transported in the blood in two forms: firstly, it is bound to hemoglobin (Hb); secondly, it is carried in simple solution in the plasma phase of the blood. The amount of O_2 carried by Hb depends on its concentration in the blood and the degree to which the Hb is saturated with O_2. The amount of O_2 that can be carried by 1g of Hb has been variously estimated to be 1.39 [6], 1.34 [7] and 1.306 [8] mL. A value of 1.34 mL of O_2 per g of Hb has been used in calculations in this paper as this gives good results at normal acid base values, and when calculating arterial/mixed venous content differences over a wide range of conditions [2]. Oxyhemoglobin saturation (SO_2) depends primarily on the PO_2 to which it is exposed. The relationship is not linear; due to the cooperativity of the Hb molecule, a graph of SO_2 against PO_2 follows a sigmoidal curve. The curve for free Hb not contained in the red cell is situated

to the left of that for intracellular Hb, indicating the higher O_2 affinity of the latter. This is due to the absence of 2,3-diphosphoglycerate (2,3-DPG) when Hb is outside the red cell envelope.

The position of the oxyhemoglobin dissociation curve also depends on a number of factors other than 2,3-DPG. The curve is moved to the right (O_2 affinity decrease) by increases in body temperature, partial pressure of carbon dioxide and hydrogen ion concentration (decrease in pH). Conversely, O_2 affinity is increased by hypothermia, hypocarbia and alkalosis. These effects can be substantial (particularly when cardiac output is decreased), and are clinically significant in the mixed venous blood where PO_2s in the range of the steep part of the dissociation curve are usually found. For instance, a 1°C rise in body temperature will decrease arterial oxygen saturation (SaO_2) by 0.3% and mixed venous saturation (SvO_2) by 0.5%. However, because the oxyhemoglobin dissociation curve is shifted to the right, the mixed venous oxygen tension (PvO_2) will actually **increase** by 5%. Hence, what is intuitively "bad" (i.e., decreases in SO_2) may actually be "good" from the standpoint of tissue oxygenation.

O_2 transport in the blood also occurs in simple solution in the plasma phase, its content being directly proportional to the PO_2. Under normal conditions of air breathing, the total content in solution is small, amounting to 0.3 mL of O_2 per 100 mL of blood per 100 mm Hg PO_2.

The total O_2 content per 100 mL of arterial blood (CaO_2) can be derived from the formula:

$$CaO_2 = (SaO_2 \times Hb \times 1.34) + (PaO_2 \times 0.3)/100$$

SaO_2 (which in the formulas is expressed as a fraction of 100 percent) can be measured in a cooximeter or derived from blood gas and acid base values by using the Kelman equation [9]. PaO_2 is the arterial partial pressure of O_2. Total mixed venous oxygen content (CvO_2) can be derived using the same formula and substituting mixed venous oxyhemoglobin saturation (SvO_2) for SaO_2 and mixed venous oxyhemoglobin tension (PvO_2) for PaO_2. Whole body oxygen delivery (DO_2) can be easily determined by utilizing cardiac output in liters per minute (CO) in the following formula:

$$DO_2 = (CaO_2 \times CO) \times 10$$

Whole body O_2 consumption (VO_2) can be calculated by multiplying the arterio-mixed venous oxygen content difference by the CO, thus:

$$VO_2 = (CaO_2 - CvO_2) \times CO \times 10$$

If body temperature and blood gas values for arterial and mixed venous blood are known, the relative contributions of Hb bound O_2 and plasma dissolved O_2 to DO_2 and VO_2 can be calculated.

The contribution of plasma-dissolved O_2 is negligible under air breathing conditions, where it contributes 1.6% of DO_2. As blood passes through the tissues, PO_2 falls and O_2 is removed from both Hb and the plasma phase. Due to the sigmoid shape of the oxyhemoglobin curve, proportionately less O_2 leaves the Hb than is taken up from plasma. Under ambient conditions the O_2 extraction from Hb is in the region of 25%; from plasma it is about 60%. This is the reason that, while plasma-dissolved O_2 accounts for only 1.6% of DO_2, it delivers 4.0% of VO_2.

When pure O_2 is inspired, PaO_2 increases to about 500 mm Hg, and plasma-dissolved O_2 will account for about 7.2% of DO_2 and will deliver 32% of VO_2. PvO_2, which is often taken as a reflection of the oxygenation state of the tissues rises from 43.5 to 51.9 mm Hg. An understanding of the importance of O_2 in the plasma phase and the increase in the fraction of VO_2 supplied by the plasma phase of blood when PaO_2 is raised is vital to an understanding of the use of PFCs in a surgical setting. We are now in a position to examine the effects of introducing a PFC emulsion into the circulation.

OXYGEN TRANSPORT BY FLUOROCARBONS

When exposed to O_2 at a partial pressure of 760 mm Hg, pure PFC liquids have O_2 solubilities that lie between 40 and 50 mL O_2 per 100 mL. For clinical and experimental use, PFC emulsions are available at concentrations varying between 20 and 90% weight-volume (w/v). PFC liquids have specific-gravities that vary between 1.7 and 2.0. This, together with the O_2 solubility of the PFC in question, determines the theoretical O_2 solubility of PFC emulsions. For example, O_2 content at 760 mm Hg varies between 4.5 mL/dL for *Oxypherol* (20% w/v emulsion of perfluorotributylamine, Green Cross Corporation) and 23.8 mL/dL for *Oxygent™ HT* (90% w/v emulsion of perflubron [perfluorooctyl bromide], Alliance Pharmaceutical Corp.). Theoretical O_2 content/PO_2 relationships for a number of PFC emulsions are presented in Fig. 1.

When PFC emulsion is infused intravenously, it mixes with blood in the circulation, and increases O_2 solubility in the plasma phase. A dose of 3 mL/kg of *Oxygent* will increase plasma solubility for O_2 from 0.3 to 0.44 mL/dL per

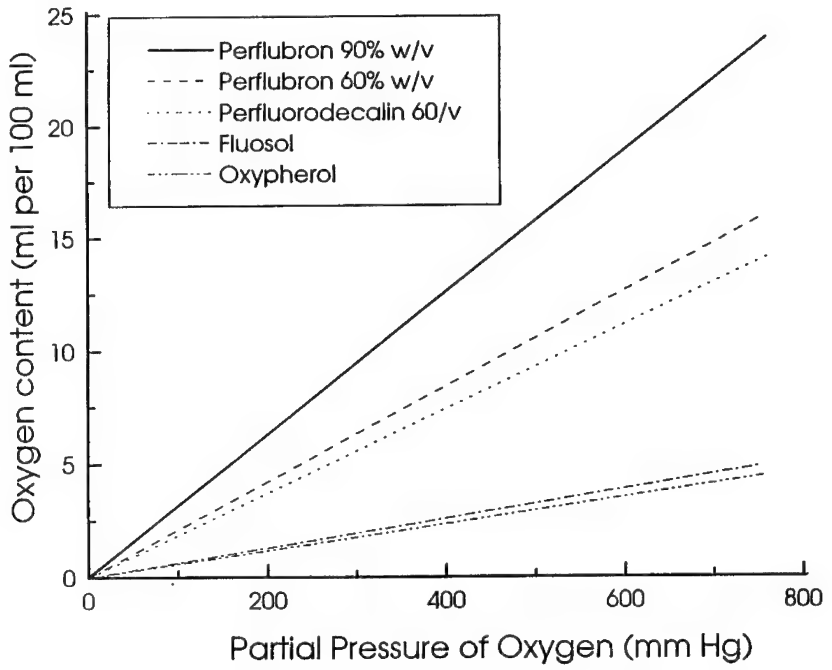

FIGURE 1: Theoretical relationships between oxygen content and partial pressure of oxygen for a number of perfluorochemical emulsions.

100 mm Hg. To obtain the same results using *Oxypherol*, a dose of 17.4 mL/kg will be necessary. Under conditions in which PFC emulsions have been infused, O_2 dissolved in the plasma phase (which now includes plasma and PFC-dissolved O_2) will provide a higher percentage of CaO_2, particularly at higher arterial O_2 tensions; this can be seen in Fig. 2 which demonstrates the effects of various doses of *Oxygent* in a patient with a Hb concentration of 14 g/dL.

It is often asked what a particular dose of a PFC emulsion represents in terms of the volume of blood that must be administered in order to create the same effect. This question cannot be answered unless several preset variables are defined. A simple in vitro calculation reveal thats in a "standard" 70 kg patient, a dose of 3 mL/kg of *Oxygent* will transport 34.4 mL of O_2 at a PaO_2 of 500 mm Hg. A unit of red cells (assumed to contain 58.5 g of Hb) will transport 78.4 mL of O_2. From this, one might conclude that the PFC emulsion is

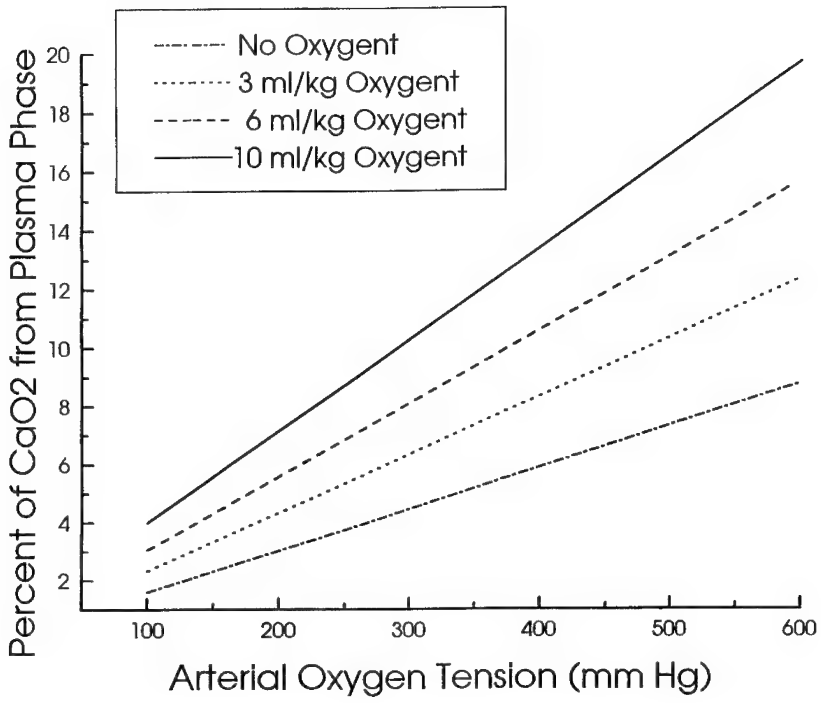

FIGURE 2: The effect of increased arterial oxygen tensions on percentage of arterial oxygen content (CaO_2) residing in the plasma phase for differing doses of *Oxygent* emulsion (90% w/v perflubron).

"equivalent" to 0.4 units of whole blood. However, as the blood passes though the tissues, only about 21% of its Hb-bound O_2 (15.7 mL) will be released, whereas 91% of the PFC-dissolved O_2 (31.2 mL) will be released. This second estimation would indicate a PFC emulsion blood "equivalency" of 2.0 units.

Ex vivo calculations such as the one above, especially if they concentrate purely on the arterial side of the circulation, are misleading, and may tend to indicate erroneously that PFC emulsions will have little effect in the hemodiluted or surgical hemorrhagic condition. When Hb concentrations are reduced, either as a result of hemorrhage and restoration of normovolemia, or as part of autologous blood strategies, there is a concomitant decrease in whole blood viscosity. As a consequence cardiac output rises and the benefit of any O_2 transporter is increased. The transporting agent is "used" more often, and the frequency with which its load of oxygen is released to the tissues is increased.

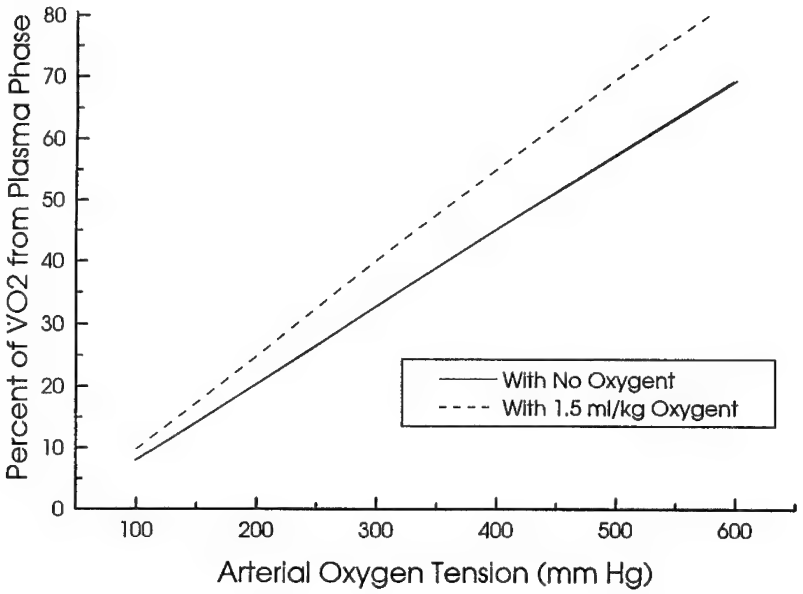

FIGURE 3: The effect of 1.5 mL/kg of *Oxygent* emulsion (90% w/v perflubron) on the percent of oxygen consumption (VO_2) delivered from the plasma phase over a range of arterial oxygen tensions.

The response in CO to the reduction in Hb concentration will vary from patient to patient; increases in CO between 0.12 and 0.55 litres per minute have been reported in a recent review [10]. In Figs. 3 and 4 the cardiac output response has been taken as 0.5 l/min increase in cardiac output for each 1 g/dL reduction in Hb concentration. Fig. 3 shows the effect of a low dose of *Oxygent* (1.5 mL/kg) and a Hb concentration of 6 g/dL on the contribution of plasma-dissolved O_2 to VO_2 over a range of arterial oxygen tensions. In Fig. 4 the corresponding mixed venous oxygen tensions are shown. As PaO_2 is increased, proportionately more O_2 is delivered to the tissues from the plasma, while at the same time PvO_2 values in the *Oxygent*-treated subject increase above those in the control subject. This implies that *Oxygent* is not only increasing plasma-compartment O_2 consumption, but that it is also disproportionately increasing PvO_2. It could be speculated that a true comparison of the two conditions might be obtained by "reducing" PvO_2 for any point to the control value by removing red cells from the circulation to reduce the O_2 availability. This, however, will further increase cardiac output and further increase the ability of the plasma

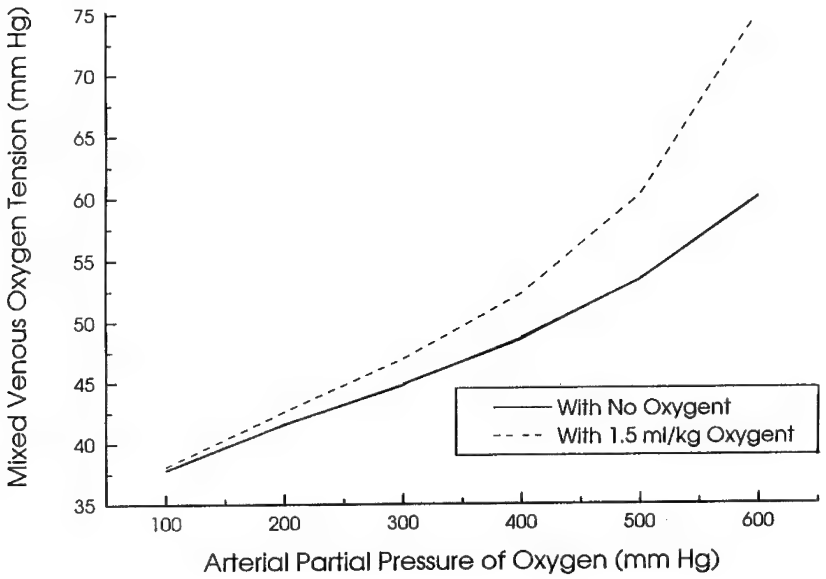

FIGURE 4: The effect of 1.5 mL/kg of *Oxygent* emulsion (90% w/v perflubron) on mixed venous oxygen tensions over a range of arterial oxygen tensions.

phase to deliver oxygen to the tissues. The whole process of calculation rapidly outgrows the potential for use of a pocket calculator. Therefore, a dedicated computer program is necessary to elucidate the intricacies of O_2 transport and delivery by PFC emulsions.

COMPUTER MODELLING

Computer algorithms have been developed to model O_2 transport and delivery to the tissues. This has been done with particular reference to the effects of progressive hemodilution during surgery in an attempt to model scenarios under which *Oxygent* might be used in the clinic. Before considering the computer model, it will be instructive to consider the possible utility of PFC emulsions in the clinic.

All artificial O_2 transporters, PFC-based or based on extracellular Hb of human, animal or recombinant origin, will be inevitably limited by their half-life in the circulation. This will range from a few hours to about a day. After a certain

length of time, it will either be necessary to administer more of the agent, or blood must be given to make up for the loss of O_2 delivery from the agent as it is removed. Repeated dosing with an O_2 transporter is unlikely to be feasible - all drugs are dose-limited and the frequency with which they can be administered is limited. It is unlikely that an O_2 transporting plasma substitute with an intravascular dwell time approaching that of red cells will be developed in the foreseeable future.

O_2 transporters could be used in a number of clinical scenarios. The two most obvious and widely applicable uses would be for the resuscitation of trauma victims and during surgery to replace O_2 transport capacity lost during bleeding. While use in trauma would appear to be very attractive, the logistics of carrying out a clinical trial under these circumstances would be very difficult. The administration of an oxygen transporter to a patient who has been prepared for surgery and who is already monitored on the operating table is much easier; this scenario is probably the one in which *Oxygent* will be employed first.

In the last few years the heightened awareness both in the lay and medical communities of the dangers of allogeneic transfusion has spurred on the development of autologous blood techniques for use during surgery. Problems arising from the use of allogeneic blood range from minor side effects, such as fever, chills and urticaria to life-threatening diseases, such as viral hepatitis and the currently fatal HIV infection. The latter, though only occurring in the USA following about 1 in 150,000 allogeneic transfusions, is very much in the public awareness and is one factor driving the increased use of autologous blood and other "bloodless" techniques in surgery. It was previously thought that allogeneic blood transfusion was a valuable and worthwhile treatment. However the American College of Physicians has recently published a paper entitled "Practice Strategies for Elective Red Cell Transfusions" in which a physician contemplating giving transfusions is urged to discuss risks and benefits with the patient, anticipate the need for autologous blood and "regard elective transfusion with allogeneic blood as an outcome to be avoided" [11].

A number of autologous blood strategies are currently employed in the perioperative period to reduce the use of allogeneic blood. In the weeks leading up to surgery, patients may predonate autologous blood, which is then used as needed during the operation to replace lost oxygen- transport capacity. This technique may be combined with administration of erythropoietin and iron to increase red cell harvest [12]. Alternatively, intraoperative normovolemic hemodilution may be practiced in which a number of units of blood are withdrawn from the patient immediately prior to surgery; in this procedure, volume deficit is made up with crystalloid or colloid, and the autologous blood

is given as required during or after the operation [10]. Other methods of obtaining autologous blood for reinfusion include collection and reinfusion of blood collected in drains in the postoperative period. Though many patients undergoing surgery can avoid allogeneic transfusions using the above autologous blood strategies, its use can never be totally averted. Even in institutions where preoperative autologous donation has become a routine, and where stringent conditions have to be met before blood transfusion is sanctioned, about 30% of patients still receive allogeneic blood [13]. It is anticipated that *Oxygent* will be given when transfusion is necessary and that autologous blood will be kept in reserve until needed.

Although blood transfusions routinely have been started as a "critical" Hb concentration or hematocrit is reached, it is clear that this is not a good measure of oxygenation of the tissues and that blood transfusion should be guided, instead, by the condition of the patient [14]. Though imperfect, PvO_2 is often taken as a measure of the level of oxygenation of the tissues. Perhaps PvO_2 should be used as a guide to transfusion and a certain value could be identified as a "trigger" for transfusion.

A computer model has been generated to calculate PvO_2 for a number of scenarios in which *Oxygent* (or any other oxygen carriers) would be used. Input variables include physical properties of the emulsion and its concentration, barometric pressure, Hb concentration, arterial and mixed venous blood gases and Hb saturations, CO and its response to hemodilution, and the patient's weight and oxygen consumption. For theoretical simulations, the bleeding rate can be entered. PvO_2 can then be calculated for a range of decreasing Hb concentrations and critical trigger points for transfusion can be identified.

Before such a computer model can be used for simulation of the clinical situation it must be validated. This may be done by comparing PvO_2 measured in pulmonary artery blood with that calculated with the program using input variables obtained from the patient at the same time that pulmonary arterial blood was sampled. A plot of measured versus calculated PvO_2 is shown for clinical data in Fig. 5. The regression coefficient is 0.93 and the regression equation is: measured PvO_2 = 1.05 * calculated PvO_2 - 0.31. Measured PvO_2 is slightly more than the calculated value, ensuring a safety margin when the program is applied to a clinical setting.

If PvO_2 is accepted as a reasonable indicator of patient safety, the question arises to what can be considered a "safe" PvO_2. Though a lot of data exists on critical oxygen delivery levels in animals, there is little to indicate what a critical PvO_2 might be in the clinical situation. What data there is would

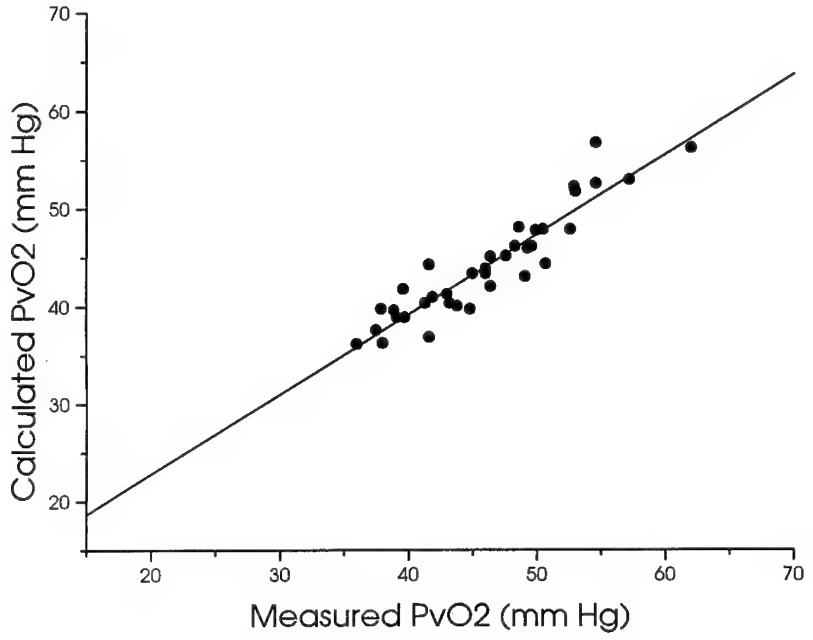

FIGURE 5: Correlation between measured mixed venous oxygen tensions (PvO_2) and those calculated on the computer simulation program; r = 0.93; measured PvO_2 = 1.05 * calculated PvO_2 - 0.31.

indicate that the level is extremely variable. For instance, in patients about to undergo cardiopulmonary bypass, critical PvO_2 varied between about 30 mm Hg and 45 mm Hg [15]; the latter value is well within the range of values found in normal, fit patients. Shunting of blood in the tissues will cause elevated levels of PvO_2, such as is found in patients in septic shock, and will result in supply dependency [16]. Nevertheless, probably "the most reliable single physiological indicator for monitoring the overall balance between oxygen supply and demand is mixed venous oxygen tension" [17].

What value for PvO_2 should be taken as a trigger for transfusion? A value of 35 mm Hg or more may be considered to indicate that overall tissue oxygen supply is adequate [17], but it must be stressed that this is implicit on the assumption of an intact and functioning vasomotor system. This level is reached at a Hb of about 4 g/dL in patients with good cardiopulmonary function; even lower

PvO_2 levels are tolerated in some patients, particularly when increased fractional inspired O_2 concentrations (FiO_2s) are employed. In the surgical situation it is necessary to maintain a good margin of safety and it is probably best to pick a PvO_2 transfusion trigger at which the patient is obviously in good condition as far as oxygen dynamics are concerned. In practice, very few patients will be monitored with a pulmonary artery catheter; thus, PvO_2 will not be available and the imperfect trigger of Hb concentration will be resurrected. If we assume that the patient is safe at this particular point, we can then calculate the effect of administration of PFC emulsions and increasing FiO_2 to 1.

The computer model predicts that a patient with good cardiac hemodilution responses (0.5 l/min cardiac output for each gm/Dl decreace in Hb concentration) will have a PvO_2 of about 44 mm Hg at a Hb of 10 g/dL when breathing air. If given O_2 to breathe, the Hb concentration can be lowered to about 1.7 g/dL before PvO_2 starts to fall below 44 mm Hg. If given 1.5 mL/kg of *Oxygent* at this point, a further 1.8 liters of blood loss can be permitted to lower PvO_2 to 44 mm Hg at an Hb of about 1.2 g/dL. The changes in PvO_2 that would occur in this example are shown in Fig. 6. The safety of the patient at extremely low levels of Hb is dependent on continuous good cardiopulmonary function, and any small decrease in CO or PaO_2 would have serious consequences. Hence, such extreme lowering of Hb is very unlikely to be permitted in routine surgical practice.

A suitable scenario for the administration of PFC's may be as follows: The clinician in charge of the patient will pick a point at which red cell transfusion should occur; this will probably be a Hb level. At this point, the patient will be given O_2 to breathe, PFC emulsion will be infused and the PaO_2 will be measured. Further surgical bleeding will be allowed and euvolemia will be maintained by infusion of crystalloids and/or colloids. PvO_2 will be allowed to fall to the value at which PFC emulsion was administered, i.e., while breathing 100% O_2. In this scenario, a margin of safety is built in by raising the PvO_2 by O_2 breathing, further O_2 delivery capacity is added then by the PFC. The initial margin of safety is maintained, and the PvO_2 is not permitted to fall below the first margin, i.e., PvO_2 at the transfusion trigger point while breathing pure oxygen.

The computer model is able to calculate the course of PvO_2 during blood loss for a number of variables such as PaO_2, cardiac output response to hemodilution, the dose of PFC and the rate of blood loss. Changes in concentration of Hb and PFC during bleeding are factored into the calculations; both are determined by the volume of blood loss, whereas PFC loss also depends on its concentration-dependent half-life in the circulation. The program

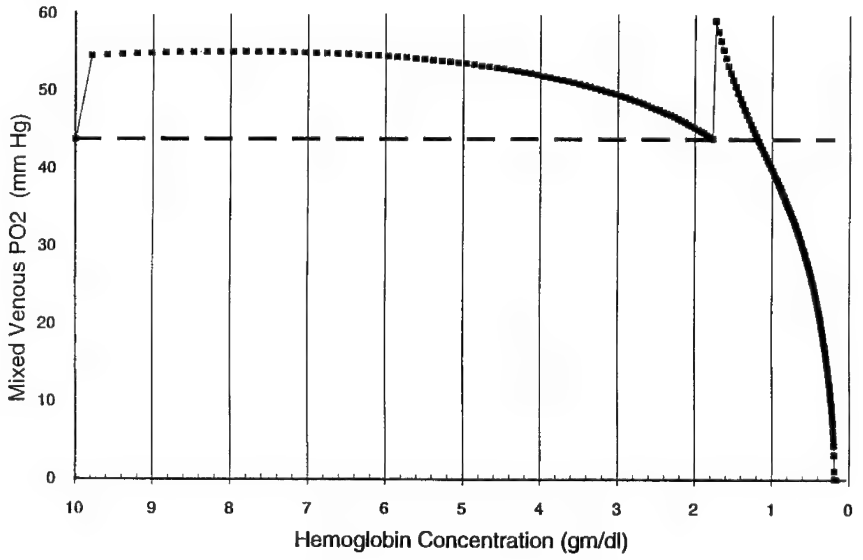

FIGURE 6: Calculated changes in mixed venous oxygen tension (PvO_2) when a patient at a Hb of 10 g/dL is given oxygen to breathe and subsequently hemodiluted. Once PvO_2 falls to the value on air at 10 g/dL Hb a dose of 1.5 mL/kg of *Oxygent* emulsion (90% w/v perflubron) is given and hemodilution is continued.

automatically produces a graph of the course of PvO_2 during progressive hemodilution for a variety of input conditions, and calculates the surgical blood loss that is permitted before transfusion is mandatory. Such a graph is reproduced in Fig. 7.

The schematic use of *Oxygent* in autologous blood strategies can be seen from the graph in Fig. 8. The graph represents two patients entering surgery with 3 units of autologous blood banked and available for use. Transfusion is to be started when Hb concentration falls to 7 g/dL. The bleeding rate is set to 1 L per hour. The control patient starts to receive autologous blood at a Hb of 7 g/dL, whereas the other receives 1.5 mL/dL of *Oxygent* and does not need autologous blood until another 2 hours and 25 minutes have passed. By this time, the control patient has used up all his autologous blood and will have started with allogeneic blood. On the other hand, the *Oxygent* patient still has 3 units of autologous blood available. In the above scenario, allogeneic blood will be necessary later in the *Oxygent* treated patient. If bleeding stops before

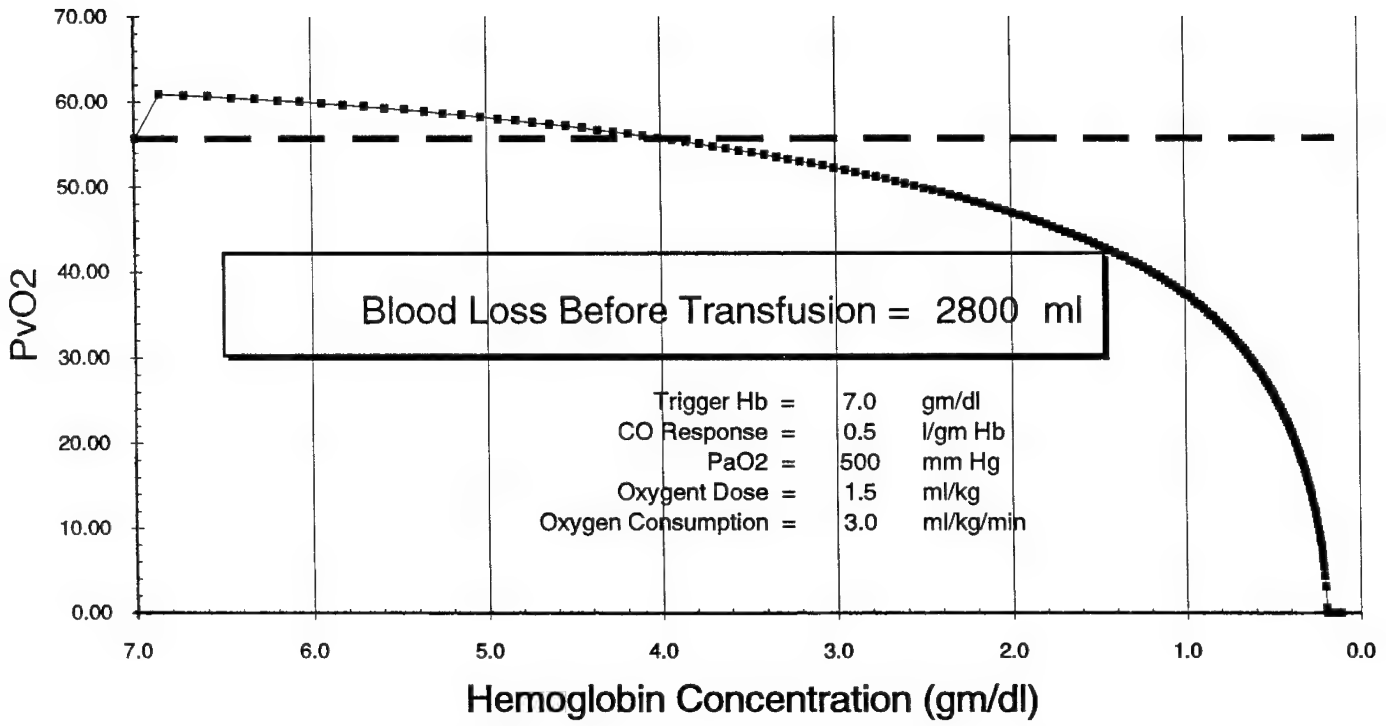

FIGURE 7: Calculated changes in mixed venous oxygen tensions when an oxygen breathing patient with a Hb of 10 g/dL is given 1.5 mL/kg of *Oxygent*. This allows the patient to loose a further 1700 mL of blood before transfusion is necessary.

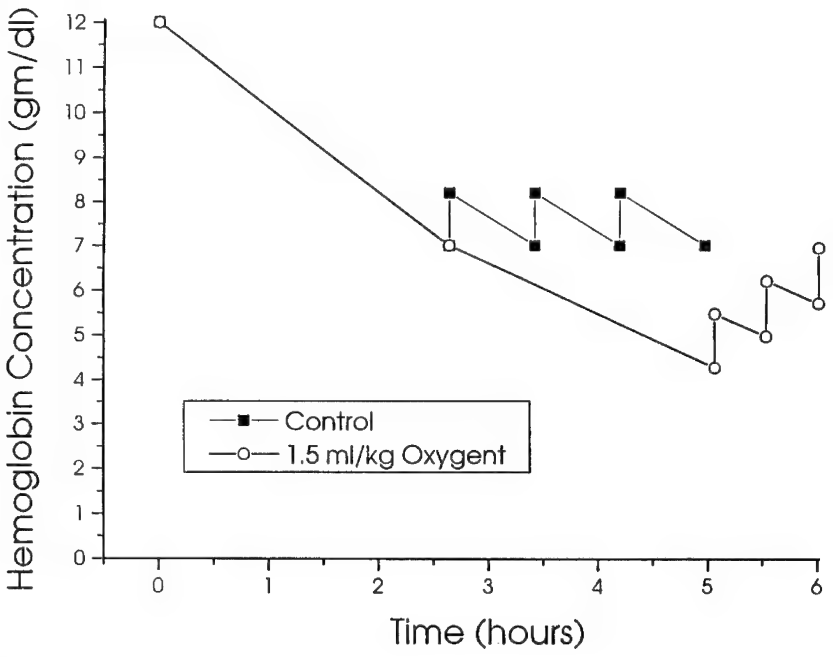

FIGURE 8: Simulated changes in Hb concentration in two patients during high blood loss surgery, both having 3 units of autologous blood available. Note the potential avoidance or reduction of allogenic blood exposure in the patient receiving *Oxygent*.

this need arises, the *Oxygent* patient will have more autologous blood in reserve or the control patient will already have received allogeneic blood. In this way the use of allogeneic blood will be reduced in patients receiving *Oxygent*.

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RANDOM CHEMICAL MODIFICATION OF HEMOGLOBIN TO IDENTIFY CHLORIDE BINDING SITES IN THE CENTRAL DYAD AXIS: THEIR ROLE IN CONTROL OF OXYGEN AFFINITY

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ABSTRACT

Knowledge of the mechanism by which chloride and carbon dioxide lower the oxygen affinity of hemoglobin may aid in the design of new blood substitutes since these allosteric regulators permit hemoglobin (Hb) to release its O_2. Stable covalent modifiers of hemoglobin, used either in a selective or a random mode, have been used to elucidate the binding sites of CO_2 or chloride. For determination of CO_2 binding, specific chemical modification of Hb by the carboxymethylation reaction was used. To identify the oxygen-linked chloride binding sites, random chemical modification of Hb was employed.

INTRODUCTION

In general, our knowledge of the mechanisms by which the oxygen bound to the heme prosthetic group of hemoglobin releases its oxygen is incomplete. It has been one of the goals of our laboratory to understand this process more fully, to determine the critical regions of the protein that govern this event, and to ascertain whether we can use this information for blood substitute research. I will describe our current understanding of two of the natural regulators of hemoglobin function, chloride and carbon dioxide, and how they influence the way in which hemoglobin releases its oxygen. The goal is that we might be able to mimic this process in blood substitute research.

When hemoglobin is present in the red cell, its oxygen affinity is low enough that it can readily release its oxygen to the tissues. This process is facilitated by the allosteric regulators present in the red cell - chloride, carbon dioxide and 2,3-DPG. However, isolated Hb in the absence of these effector molecules has such a high oxygen affinity that it cannot be used to deliver oxygen efficiently. It has been known for some time that by addition of carbon dioxide, DPG, or chloride, the increased oxygen affinity of isolated hemoglobin can be lowered to approach that of whole blood [1]. Since chloride is present in plasma, a hemoglobin-based blood substitute should have most of its chloride binding sites unencumbered in order to interact with chloride and attain a lower oxygen affinity. The same is true for CO_2, either

bound covalently as the carbamate or electrostatically as bicarbonate. It is conceivable that there are different degrees or strengths of chloride or bicarbonate binding sites so that when the primary site is blocked, the secondary or tertiary sites become operative. This possibility is just now being addressed in hemoglobin research.

METHODS

In our studies on the binding of carbon dioxide, we have used the carboxymethyl (Cm) derivative [2]. The carbamino adduct of carbon dioxide with hemoglobin is extremely labile and difficult to work with in the laboratory. However, the carboxymethyl analog that we have prepared is very stable and binds to the same sites on hemoglobin as carbon dioxide. For studies on chloride binding sites, we have used an acetylation (Ac) reaction with the mild bifunctional agent, methyl acetyl phosphate [3,4].

Specific Carboxymethylation of Hb - Reductive carboxymethylation with glyoxylate and sodium cyanoborohydride was used to achieve N-carboxymethylation at the amino groups [2,5], which is different from S-carboxymethylation of sulfhydryl groups. The reaction proceeds under mild conditions at neutral pH at room temperature. The products were purified by conventional chromatography and were shown by biochemical analysis to have the carboxymethyl group at the N-termini of the Hb chains.

To prepare hybrid carboxymethylated tetramers, *i.e.*, those in which the N-terminus of either the α or the β chain was carboxymethylated and the other chain had an unblocked N-terminus, a strategy that was employed successfully was recombination of equivalent amounts of carboxymethylated α or β chains with unmodified β or α chains, respectively. It is our experience that this approach is preferable to attempts at modifying selectively either the α or β subunit in the intact tetramer. The specifically N-carboxymethylated Hb hybrid tetramers prepared in this manner have been shown to be pure and fully functional [5].

Random Acetylation of Hb - In an effort to locate all of the functional, i.e. oxygen-linked chloride binding sites of Hb, an approach that we refer to as *random* chemical modification was used [6]. The reagent that we chose for this purpose was methyl acetyl phosphate (MAP), which we had used previously in a selective manner to label the amino groups in the DPG site of hemoglobin [3]. It is a bifunctional reagent with a phosphate group at one end and acetyl donor at the other. In the present study, we used radiolabeled MAP to identify those chloride binding sites that were oxygen-linked, i.e. acetylated to a greater extent in the deoxy form compared with the oxy form. To identify these sites we employed peptide mapping with a tandem treatment of trypsin and chymotrypsin. The amount of ^{14}C-radiolabel incorporated into a peptide was taken as an approximation of the extent of chloride binding.

RESULTS

The Effect of Carboxymethylation on the Functional Properties of Hb - The hybrid Hb carboxymethylated on the N-termini of both α chains, $\alpha_2^{Cm}\beta_2$, had an intrinsic oxygen affinity

Table I. Functional Properties of Modified Hemoglobin Derivatives

Hemoglobin Derivative	P_{50}	Hill coefficient	Alkaline Bohr Value[a]
Carboxymethylated Hb	(mm Hg)	(n)	(H^+ released/tetramer)
$\alpha_2\beta_2$ (unmodified)	7	2.4	2.12
$\alpha_2^{Cm}\beta_2$	12	2.4	2.00
$\alpha_2\beta_2^{Cm}$	17	2.4	1.52
$\alpha_2^{Cm}\beta_2^{Cm}$	37	2.4	1.52

[a] The alkaline Bohr effect was measured in the presence of 0.1M chloride.

(P_{50} = 12 mm Hg) that was lower than that of the native protein (P_{50} = 7 mm Hg) (Table I). Addition of 2,3-DPG to this hybrid lowered its oxygen affinity about 4-fold, to 48 mm Hg. It had a reduced response to added chloride because a major chloride binding site comprising Val-1(α) already had an anion covalently attached; all other chloride binding sites in this hybrid, especially those involving the β chains, were unmodified and therefore free to interact with added chloride [2,5].

The hybrid carboxymethylated on the N-termini of both β chains, $\alpha_2\beta_2^{Cm}$, had an oxygen affinity (P_{50} = 17 mm Hg) that was also lower than that of the unmodified tetramer (Table I). The presence of the carboxymethyl group protruding into the cleft between the two β chains, as shown by X-ray diffraction analysis, did not prevent further lowering of the oxygen affinity by 2,3-DPG (maximum P_{50} = 25 mm Hg). The addition of chloride to $\alpha_2\beta_2^{Cm}$ resulted in a significant lowering of the oxygen affinity due mainly to the binding of the chloride anion to the region around the N-terminus of the α-chain, which was free in $\alpha_2\beta_2^{Cm}$ as well as to other sites (see below) [2,6].

The hybrid carboxymethylated on all four N-terminal residues, $\alpha_2^{Cm}\beta_2^{Cm}$, had an oxygen affinity that was considerably lower (P_{50} = 37 mm Hg) than the additive effects of this modification at the N-termini of the individual α and β chains (Table I). The addition of 2,3-DPG to this hybrid resulted in a further lowering of the oxygen affinity (P_{50} = 50 mm Hg). Both $\alpha_2\beta_2^{Cm}$ and $\alpha_2^{Cm}\beta_2^{Cm}$ retained some response to 2,3-DPG probably because the Cm group did not fully occupy the DPG site. The hybrid $\alpha_2^{Cm}\beta_2^{Cm}$, which had two of its major anion-binding regions covalently occupied with a negatively charged anion, underwent further lowering of its oxygen affinity by chloride, to the extent of about 20-25% of the total chloride effect (see below) [2,6].

For all of the hybrids studied (except for $\alpha_2^{Cm}\beta_2^{Cm}$ at low oxygen tensions), the degree of cooperativity was unaffected by the modification; n values remained at 2.4 (Table I) [5].

Table II. Differences Between Selective and Random Protein Modification.

Selective	**Random**
Attempt to modify all molecules	Limit number of modified molecules
Isolate a homogenous product	Mixture of modified molecules
Quantitative goal: unique modified site	Qualitative goal: identification of major and minor sites
Possible total or partial loss of protein function	Retention of protein function

This finding argues against significant distortion of subunit contacts after introduction of the negatively charged carboxymethyl group.

Alkaline Bohr Effect - The alkaline Bohr effect was not lowered to a significant extent in the derivative with the carboxymethyl group on the N-terminus of the α-chain (Table I) [5]. This result is consistent with the suggestion that if the charge on the N-terminal amino group is maintained, then it can still function as a Bohr group. However, with the carboxymethyl group was on the N-terminus of the β-chain, the alkaline Bohr effect was reduced by about 25% [5]. This reduction could be due to the interaction of the carboxymethyl group on the N-terminus of the β-chain with Lys-82(β), as elucidated by x-ray diffraction analysis [5]. In the presence of 0.1 M sodium chloride, the alkaline Bohr coefficient is about 20% higher than in its absence for all hybrids including unmodified hemoglobin (Table I). This effect is likely due to the interaction of chloride with those NH_2 groups partly responsible for the alkaline Bohr coefficient.

Blocking of Major Chloride Binding Sites - A hybrid tetramer, which had the N-terminus of the α-chain blocked by an uncharged carbamyl group and a β-chain from hemoglobin Providence in which Lys-82(β) was substituted by an asparagine, was constructed [7]. In this hybrid, only about 20% of the remaining oxygen-linked chloride binding sites remained, consistent with the findings above on the effect of chloride on $\alpha_2^{Cm}\beta_2^{Cm}$. For several years, we attempted to locate this residual 20% chloride effect by conventional approaches, but it was not clear whether it was simply due to an accumulation of small contributions from different amino acid residues or whether it arose from discrete sites. Recently, we decided to approach this question by using a variation of the chemical modification technique - random chemical modification [6].

Random vs Selective Chemical Modification - In selective or specific chemical modification of proteins, an attempt is made to achieve modification of all molecules, whereas in random chemical modification, the objective is to limit the number of modified molecules. A comparison between random and selective modification is shown in Table II [6].

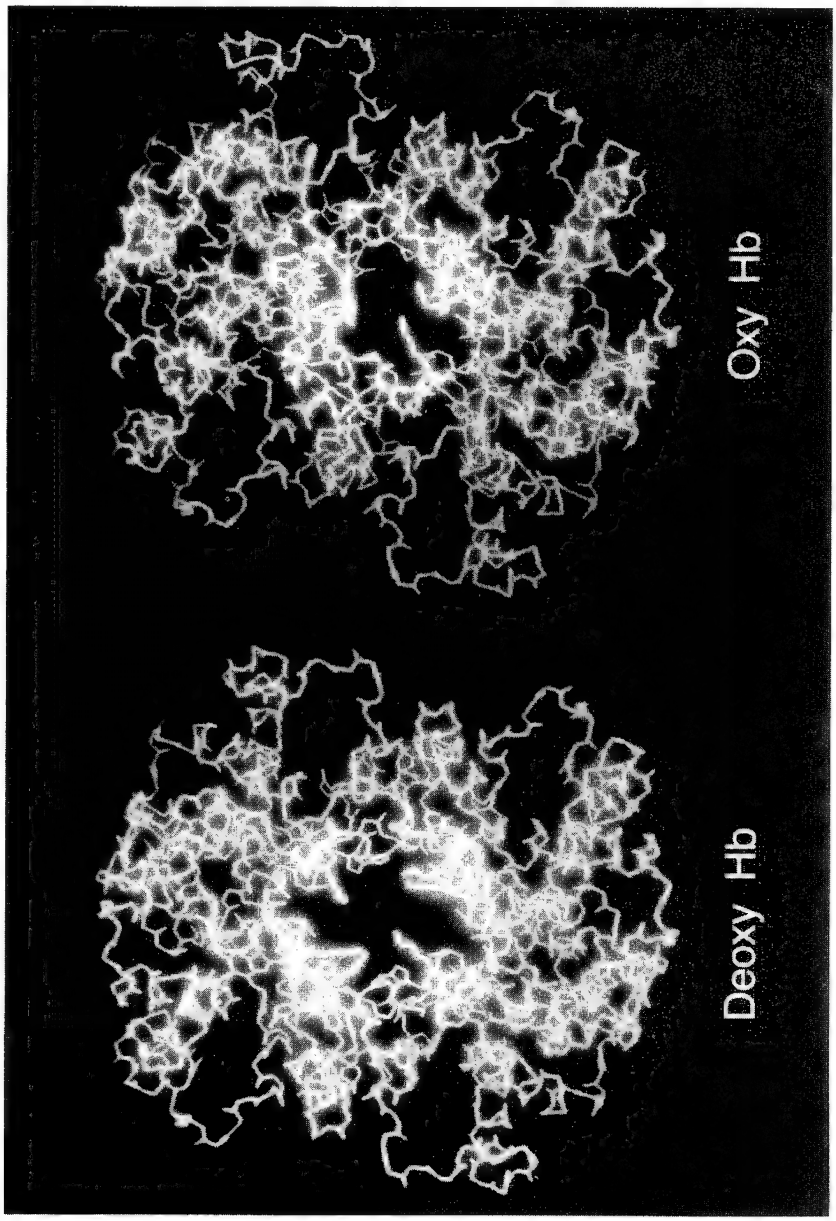

Figure 1. View of deoxy Hb (left) and oxy Hb (right) into the central dyad axis. The chloride binding sites are highlighted.

With the selective chemical modification approach, a homogeneous product is usually isolated, whereas in random chemical modification, one works with a mixture of modified molecules. The goal in the selective chemical modification approach is a quantitative one, i.e. to obtain the unique modified site, but in random chemical modification, the goal is qualitative, i.e. to identify major and minor chloride binding sites. Sometimes in selective chemical modification, there is a total or partial loss of protein function, but in the random modification approach, retention of protein function is desirable to ensure that the modification has not affected the function of the protein. For Hb, this is readily achieved by determination of the Hill coefficient, i.e. the degree of subunit cooperativity.

We used bovine hemoglobin since it was more responsive to chloride than human hemoglobin [3,4,6]. We removed as much of the bound chloride as possible so that we would have a better chance of identifying even minor chloride binding sites. We demonstrated that randomly acetylated hemoglobin mimicks unmodified hemoglobin because only a fraction of the molecules are acetylated on any one site. Hence, the decrease in oxygen affinity upon addition of chloride is parallel for both randomly acetylated and unmodified hemoglobin [6]. The Hill coefficient value for randomly acetylated hemoglobin is 2.2 compared with 2.3 for unmodified hemoglobin (Fig. 2). Two oxygen-linked chloride sites on the α-chain (Val-1 and Lys-99) and three sites on the β-chain (Met-1, Lys-81 and Lys-103) were identified. Some of these sites were already known from our earlier results as well as those of other investigators. Lys-99(α) and Lys-103(β) reside along the sides of the central dyad axis and connect the two major chloride binding sites at Val-1(α) and Lys-82(β) at opposite ends of the molecule.

Molecular modeling techniques of deoxy hemoglobin have shown that the oxygen-linked binding sites are symmetrically related, i.e. opposite one another (Fig. 1). It is likely that they would then repel each other and hold the central dyad axis in an open conformation in the presence of chloride. Indeed, it has been known for many years that the central dyad axis has a more open conformation in deoxy hemoglobin than in oxy hemoglobin [8]. It is possible that the crosslink between the two Lys-99(α) sites [9] has a low oxygen affinity for this reason. Other studies [10,11] have shown that when large organic anions are bound to this site, the oxygen affinity is lowered. Perhaps these compounds act by preventing the constriction of the central dyad axis, and thereby increasing the P_{50}. A goal of future studies is to mutagenize certain sites around this area to test this proposal, and to make new chloride binding sites by site-directed mutagenesis using the yeast expression system [12].

Another important part of blood substitute research is to ensure that the hemoglobin tetramer does not dissociate into its dimers, which are rapidly cleared by the circulation. We have addressed this question and we use the crosslinking reagent, diisothiocyanatobenzene sulfonic acid (DIBS), to crosslink the two N-terminal residues of the α-chains of hemoglobin [13]. We obtained several products, which we purified by conventional chromatography. Using a variety of biochemical analysis, such as amino acid analysis, mass spectrometry, protein sequencing, HPLC, SDS gel electrophoresis, we showed that in the major product, the crosslink was between the N-terminals of the α-chain. When we compared the plasma retention time of

the DIBS-crosslinked hemoglobin with that of carboxymethylated (Cm) hemoglobin, we found that the Cm hemoglobin had a retention time of about 0.65 hrs which is about the same as unmodified hemoglobin [13]. The DIBS-crosslinked hemoglobin had a plasma survival time of just over 3 hrs, which was considerably improved over that of Cm-Hb. We concluded from this study that it was the ability of hemoglobin to dissociate and not its P_{50} that were important in keeping Hb in the plasma since DIBS crosslinked hemoglobin does not have a decreased P_{50}. Future studies along these lines will be aimed at achieving even higher molecular weight hemoglobins so that we might determine the minimum molecular weight for crosslinked hemoglobin to maintain it in the circulation for the maximum desirable period.

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HEME AND THE VASCULATURE: AN OXIDATIVE HAZARD THAT INDUCES ANTIOXIDANT DEFENSES IN THE ENDOTHELIUM

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ABSTRACT

Heme proteins transport oxygen and facilitate redox reactions. Heme, however, may be dangerous, especially when free in biologic systems. For example, iron released from hemoglobin-derived heme can catalyze oxidative injury to neuronal cell membranes and may be a factor in post-traumatic damage to the central nervous system. We have shown that heme catalyzes the oxidation of low density lipoproteins which can damage vascular endothelial cells. The endothelium is susceptible to damage by oxidants generated by activated phagocytes, and this has been invoked as an important mechanism in a number of pathologies including the Adulte Respiratory Distress Syndrome (ARDS), acute tubular necrosis, reperfusion injury and atherosclerosis. Because of its highly hydrophobic nature, heme readily intercalates into endothelial membranes and potentiates oxidant-mediated damage. This injury is dependent on the iron content of heme and is

completely blocked when concomitant hemopexin is added. Ferrohemoglobin, when added to cultured endothelial cells, is without deleterious effects, but if oxidized to ferrihemoglobin (methemoglobin), it greatly amplifies oxidant damage. Methemoglobin, but not ferrohemoglobin, releases its hemes which can then be incorporated into endothelial cells. Cultured endothelial cells, when exposed to methemoglobin but not ferrohemoglobin, cytochrome c or metmyoglobin, potentiate this oxidant injury. Stabilization of the methemoglobin by cyanide, haptoglobin or capture of the heme by hemopexin abrogates this effect. Paradoxically, more prolonged exposure of endothelium to heme or methemoglobin renders them remarkably resistant to oxidant challenge. Endothelium defends itself from heme by induction of the heme degrading enzyme heme oxygenase and the concomitant production of large amounts of the iron binding protein ferritin. The ferritin content of endothelial cells is inversely proportional to their susceptibility to oxidant damage under a wide range of experimental conditions. We conclude that, acutely, delivery of free heme to the vasculature is hazardous by sensitizing endothelial cells to oxidant damage while chronic exposure upregulates their defense of heme oxygenase and ferritin.

INTRODUCTION

Our laboratory has focused upon the role of oxygen radicals derived from phagocytes and other sources in mediating vascular endothelial damage. Free radicals have been implicated to underlie the pathophysiology of numerous disease states including acute renal failure associated with ischemia reperfusion, myocardial infarction, adult respiratory distress syndrome, arthritis and hemorrhagic shock [1]. These free radicals are highly reactive species capable of adding or taking electrons from biological molecules such as lipids, proteins or even DNA and setting up a chain reaction that propagates more radicals. Transition metals including iron, copper and nickel reduce hydrogen peroxide to generate the very reactive free radical, hydroxyl radical, via the Fenton reaction. *In vivo* hemoglobin is the most abundant source of iron and can catalyze oxidant damage. Iron spontaneously released from hemoglobin can catalyze oxidative damage to neuronal cell membranes and may be a factor in post-traumatic damage

to the central nervous system [2]. We have focused on heme as a hydrophobic iron chelate which can catalyze the peroxidation of low density lipoproteins and potentiates oxygen radical mediated injury to endothelial cells [3,4]. In our studies, we demonstrate that heme promotes oxidant-mediated endothelial damage but also can induce cytoprotective proteins in the endothelium, heme oxygenase and ferritin, which can prevent this injury [5]. The induction of heme oxygenase and ferritin is dependent on the release of heme from hemoglobin, especially when hemoglobin is oxidized to methemoglobin [6]. That these cytoprotective mechanisms are operative *in vivo* are demonstrated in a rat model of kidney injury due to rhabdomyolysis [7]. Induction of ferritin and heme oxygenase in the kidneys of rats protects them against subsequent myoglobin-induced renal injury. This paper will review previously published observations.

MATERIALS AND METHODS

Endothelial cell cultures

Human umbilical vein endothelial cells and porcine aortic endothelial cells were grown to confluence as previously described [8].

Endothelial cell cytotoxicity assays

Confluent endothelial cells were radiolabelled with ^{51}Cr and cytotoxicity induced by hydrogen peroxide, hypoxanthine/xanthine oxidase or activated neutrophils measured as previously described [4].

Heme oxygenase and ferritin assays

Heme oxygenase activity in endothelial cells was measured by bilirubin generation [9]. Endothelial cells ferritin content was measured by ELISA [5]. Heme oxygenase mRNA were analyzed in endothelial cells by Northern analysis [5].

Rat rhabdomyolysis studies

Details of the induction of rhabdomyolysis by glycerol and biochemical measurements in these animals and their kidneys were previously described [7].

RESULTS AND DISCUSSION

When endothelial cells are incubated with the hydrophobic iron chelator hemin substantial quantities of heme rapidly accumulate within the cells and cell membranes. Hemin itself is not dangerous to the cultured endothelial cells; however, minute quantities of hydrogen peroxide or granulocyte-derived oxidants, themselves are not harmful in these concentrations, added to these heme loaded cells, cause marked lysis. This heme augmentation of cytolysis parallels lipid peroxidation which can be inhibited by the hydrophobic oxygen radical scavenger/iron chelator U74500A [10]. The iron moiety of heme is critical to oxidant sensitization because neither iron-free protoporphyrin IX nor tin protoporphyrin are able to sensitize endothelial cells to hydrogen peroxide or activated granulocytes. The hydrophobicity of heme is critical for uptake in the endothelium. Hydrophilic ferriprotoporphyrins such as iron deutero-IX,2,4-bis sulfonate, which substitutes sulfonate for vinyl, do not enhance oxidant mediated cytotoxicity. The heme binding protein hemopexin blocks endothelial uptake of heme and prevents heme augmented oxidant cytolysis if hemopexin is added simultaneously and stoichiometrically with hemin. These findings indicate that acute exposure of the vasculature to the hydrophobic iron chelate hemin allows intercalation into cell membranes and may be important in the genesis of a number of clinical conditions including acute renal failure associated with intravascular hemolysis or myoglobinemia or ischemia reperfusion injury in which release of reactive iron has been demonstrated.

Does hemoglobin, after infusion or release by sheared red blood cells, sensitize endothelial cells? Initial studies involving addition of hemoglobin to endothelial cells followed by oxidant stress did not increase their cytotoxicity [6]. However, oxidation of hemoglobin to methemoglobin which destabilizes the protein allows the highly hydrophobic heme group to be readily detached [11]. Bunn and Jandl showed that hemes are detachable from hemoglobin and can exchange between globin and other proteins such as albumin. This occurs more rapidly if hemoglobin is in the oxidized methemoglobin state but is preventable by

stabilizing the heme protein with cyanide. In our studies, stabilization of the heme group in methemoglobin by cyanide, capturing released heme by hemopexin or binding methemoglobin to haptoglobin prevented oxidant sensitization.

The vascular endothelium, because of its continuous contact with circulating red cells, would thus be at risk from exogenous heme exposure. Therefore, adaptive strategies must be inducible when oxidant stress is excessive. In our studies, when endothelial cells are exposed to heme for a prolonged time period (16 hours), the cells become highly resistant to oxidant-mediated injury and to the accumulation of endothelial lipid peroxidation products. This protection is associated within four hours of the induction of heme oxygenase mRNA. After 16 hours, heme oxygenase and ferritin have increased 50-fold and 10-fold respectively. Hemoglobin did not induce heme oxygenase mRNA nor did cytochrome c or metmyoglobin. In contrast, methemoglobin induced heme oxygenase mRNA activity as well as ferritin protein in endothelial cells. Methemoglobin induction of heme oxygenase and ferritin was blocked by liganding it with cyanide or haptoglobin or capturing the released heme by hemopexin. Prolonged exposure of endothelium to methemoglobin similarly made them resistant to oxidant mediated cytolysis. The differential induction of heme oxygenase and ferritin pointed to ferritin as the critical cytoprotectant. Ferritin inhibits oxidant mediated cytolysis in direct relation to its intracellular concentration. Apoferritin, when added to cultured endothelial cells, is taken up in a dose dependent manner and appears as cytoplasmic granules by immunofluorescence. In a similar dose responsive manner, added apoferritin protects endothelial cells from oxidant mediated cytolysis. Conversely, a site directed mutant of ferritin (heavy chain Glu 62 $\rightarrow$ Lys; His 65 $\rightarrow$ Gly), which lacks ferro-oxidase activity and is deficient in iron sequestering capacity, is completely ineffectual as a cytoprotectant [12]. We conclude that endothelium and perhaps other cell types may be protected from oxidant damage through the endogenous iron-chelator ferritin, which can bind 4500 potential iron atoms per ferritin molecule as Fe^{3+}. The heavy chain of ferritin, by manifesting ferro-oxidase activity, is critical for this protection by allowing ferritin stored iron to

resist cyclical reduction oxidation which tends to propagate and amplify oxidative damage. Are these findings relevant to *in vivo* oxidant mediated endothelial injury? In a model of glycerol-induced rhabdomyolysis in the rat as well as the clinical syndrome, the kidneys are the targets of heme protein mediated injury. The exposure of the kidney to heme proteins such as myoglobin or hemoglobin induces the heme degrading enzyme, heme oxygenase, as well as synthesizing the iron chelator ferritin. Unlike control animals, rats, when infused with rat hemoglobin at a dose of 30 mg/100 gm body weight prior to glycerol induced rhabdomyolysis, did not develop renal failure or die. Thus, induction of heme oxygenase coupled with ferritin synthesis protects against heme protein mediated injury. Whether such cytoprotective strategies would prevent the toxicity due to hemoglobin based blood substitutes is yet to be determined.

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THE DESIGN AND DEVELOPMENT OF IMPROVED FLUOROCARBON-BASED PRODUCTS FOR USE IN MEDICINE AND BIOLOGY

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ABSTRACT - Fluorocarbons and other highly fluorinated materials offer considerable potential in diagnosis and therapeutics due to their unique physical properties, chemical inertness, capacity to transport oxygen and drugs, and ability to function as contrast agents. Applications such as hemodilution and organ preservation, cancer diagnosis and chemotherapy, x-ray imaging of the lymph nodes and magnetic resonance imaging of the GI tract, cardioplegia and reperfusion, the treatment of myocardial ischemia and respiratory distress syndrome, as well as drug delivery, all obviously require different product characteristics, calling for an array of products which may range from different neat fluorocarbons to diversely formulated emulsions, or fluorinated vesicles. Substantial progress has been made in terms of emulsion efficacy and stability. Stable, ready-to-use, concentrated, though fluid, injectable emulsions have now been developed. Small doses of such emulsions were demonstrated to be highly efficient in tissue oxygenation. Commercial-scale manufacturing including heat sterilization of these emulsions have been achieved. Some of the side-effects, which generally relate to the normal response of the organism to injected particles, have been reduced, and their mechanism determined. Further efforts will

undoubtedly be devoted to understanding and adjusting emulsion properties for optimal efficacy in each identified application and to maximizing benefit *vs* side-effect ratio. Our ability to modulate *in vivo* recognition, intravascular persistence and subsequent biodistribution of fluorocarbon droplets, vesicles and other particulate matter in the organism is still in its infancy. Proper control of these characteristics would further extend the potential of such products for medical uses. It is essential that no effort be spared to increase our general understanding of their physicochemical properties and *in vivo* "physiology".

AN EXTENDED POTENTIAL OF APPLICATIONS

One major objective of research on fluorocarbon products for biomedical applications in medicine is, of course, the development of oxygen carriers which could serve as temporary red blood cell substitutes [1-3]. This is an objective that we have in common whether we work on fluorocarbon-based products or on hemoglobin-based products. We certainly also share some fundamental questions about tissue oxygenation, efficacy-testing protocols, administration conditions, understanding and control of reactions of the organism to the administration of foreign material, especially when in the form of particulates, etc. While efforts toward developing fluorocarbon-based red blood cell substitutes are more vigourous than ever, numerous other potential applications of fluorocarbons in medicine are also being explored [1,4]. These applications exploit some of the unique chemical and physical properties of fluorocarbons (Table I) [5,6].

Injectable oxygen carriers

Researchers are taking advantage of the exceptionally high solubility of gases in fluorocarbons by developing injectable products to deliver oxygen to tissues in situations where blood flow is compromised and/or blood transfusion ineffective, contraindicated, or undesired. Such products, in which the fluorocarbon is formulated as an emulsion, should be valuable in perioperative hemodilution; for the perfusion of ischemic tissues (for example the myocardium after infarction) possibly in conjunction with a clot-dissolving drug; for priming cardiopulmonary bypass machines; for use during cardiovascular surgery as cardioplegic and reperfusion solutions; as an adjunct during percutaneous transluminal coronary

TABLE I : Basic characteristics of fluorocarbons that may be used as injectable gas carriers

- ***SYNTHETIC*** *- LARGE SCALE FEASIBILITY*
  WELL ESTABLISHED PRODUCTION TECHNOLOGY
- *STABLE - STERILIZABLE - NO INFECTIOUS RISK*
- ***DISSOLVED*** O_2 *(no chemical binding)* → O_2 *READILY AVAILABLE TO TISSUES*
  HIGH EXTRACTION RATE & RATIO
- ***LINEAR*** O_2 ***vs*** pO_2 ***UPTAKE*** *- NO SATURATION*
  INCREASED O_2*-DELIVERY SIMPLY BY INCREASING* PO_2
- *HIGH* CO_2 *SOLUBILITY*
- *GAS SOLUBILITY* ↑ *WHEN TEMPERATURES* ↓
- ***NO POSSIBILITY OF CHELATION*** *OF CO, NO (nitric oxide)*
- *INSOLUBLE IN WATER - NO DIFFUSION IN TISSUES*
- ***NOT METABOLIZED*** *- NO METABOLITE-RELATED TOXICITY*
- *EXCRETED BY EXHALATION*
- ***MODULABLE LIPOPHILICITY*** *i.e.* ***v.p. & EXCRETION***
- *LOW SURFACE TENSION*
- ***LOW KINEMATIC VISCOSITY***
- *HIGH DENSITY*
- *HIGH COMPRESSIBILITY, LOW ACOUSTIC VELOCITY*
- *NO PROTONS -* ^{19}F *PROBE*
- *Can have POSITIVE SPREADING COEFFICIENT*
- *Can be* ***RADIOPAQUE***

angioplasty (PTCA) procedures; for sensitization of cancerous tumor cells to radiation and chemotherapy; for hematopoietic stimulation; for treatment of sickle cell anemia; for the preservation of organs destined for transplantation, etc.

Use of the oxygen carrier in conjunction with blood predonation and perioperative acute normovolemic hemodilution in order to conserve blood for use at the end of surgery or post-operatively is one of the first clinical applications targeted [7]. The objectives here are to provide an increased margin of safety for

the patient and to minimize the potential need for eventual allogenic blood transfusion. In this application, a portion of the patients' blood is withdrawn prior to surgery, replaced by both a volume expander and the oxygen-carrier. This blood is then available to be reinfused, when needed, during or after the procedure. Fluorocarbon emulsions allow more extensive hemodilution at a lower risk for the patient and minimize the need for allogenic blood. They should therefore play a fundamental role as part of general autologous blood conservation strategies aimed at the reduction of diseases transmitted by blood transfusion. Demonstrating the effectiveness of a blood substitute is not an easy task; it should be facilitated in hemodilution trials [8]. A concentrated emulsion has also been announced to commence clinical trials as an adjunct to cancer chemotherapy.

Diagnosis

In a different area, diagnostic medicine, fluorocarbon-based products can provide unique contrast enhancement properties. Some of these products are applicable in all of the major imaging modalities: magnetic resonance imaging (MRI) - because of the absence of protons, fluorocarbons produce areas of "signal-void"; ultra-sound - because droplets in some fluorocarbon emulsions reflect sound waves differently from tissues; conventional x-ray radiography and computed x-ray tomography (CT) - if the fluorocarbon is made radiopaque by the presence of a heavy halogen atom, as for example in perfluorooctyl bromide ($C_8F_{17}Br$, perflubron) [9].

A neat fluorocarbon used as an oral contrast agent during MRI to distinguish the gastrointestinal tract from adjacent tissues and detecting possible pathology has completed Phase III clinical trials and awaits approval by the United States' Food and Drug Administration (FDA) [10]. An externally applied fluorocarbon device, now commercially available, reduces magnetic susceptibility differences, thereby improving the quality of fat saturation techniques, and consequently of magnetic resonance imaging [11]. The imaging of lymph nodes with CT after subcutaneous injection of an emulsion of perfluorooctyl bromide may allow the early, non-surgical detection of cancer in lymph nodes [12]. This product, which is in Phase II trials, could play an important role in diagnosing the stage of development of cancer, hence determining the most appropriate treatment at an early stage. Another

perflubron emulsion, now being studied in cancer patients, can be administered intravenously as a means of detecting cancer in blood-rich organs such as liver and spleen [9,13]. A key advantage of such emulsions over water-soluble iodinated contrast agents is that they remain in the blood pool for several hours, facilitating the procedure and allowing the differentiation of very small tumors from normal tissues and blood vessels.

Liquid ventilation and miscellaneous applications

Other new important applications of fluorocarbons in therapeutics are based on partial [14] or total [15] liquid ventilation of the lungs. These procedures may be used as a treatment of the respiratory distress syndrome (RDS), a severe condition with high mortality and morbidity. The neat fluorocarbon is administered intratracheally into the impaired lungs, spreads rapidly and uniformly into the alveoli, lowers the airway pressure necessary to ventilate the lungs, and facilitates gas exchange. Preclinical work indicates adequate gas exchange and good tolerance in various animal species. The fluorocarbon can also be employed to deliver drugs through the pulmonary route [16] and the use of a radiopaque fluorocarbon allows the imaging of pulmonary structure [17].

Still further applications of fluorocarbons or highly fluorinated materials include their uses as a tamponade in retinal surgery, as components of drug delivery systems such as liposomes with modified membrane permeability, and in cell and tissue culture [1,4].

WHICH PRODUCTS ?

In view of this diversity of applications, an array of tailor-made products is desirable. They may be based on various neat fluorocarbons and other highly fluorinated materials, diversely formulated emulsions, fluorinated vesicles (which could, among others, encapsulate hemoglobin), etc (Table I).

Key objectives in the development of such products include the selection of appropriate, industrially feasible components; formulation for optimal safety and efficacy in each application; extended storage stability and convenient use; large-scale manufacturing capability; assessment, through preclinical and clinical studies, of uses, effective doses, safety and efficacy; understanding, minimization and

control of side-effects; approval by the regulatory agencies, etc. These objectives obviously require that we continuously improve our fundamental knowledge of the physicochemical properties of these products and their behavior and effects in the organism.

An early fluorocarbon emulsion, *Fluosol®* (Green Cross Corp., Osaka, Japan) developed in the late seventies [18], has been licensed by the FDA as a means of protecting the myocardium during PTCA (balloon angioplasty) and is now commercially available. Similar preparations have subsequently been developed in Russia (Ftorosan and Perftoran) [19] and China [20]. These "first generation" products have their shortcomings, which include limited application, insufficient stability and slow excretion. Since the time they were developed our knowledge about fluorocarbons and fluorocarbon emulsions has greatly improved.

This review will briefly summarize the progress that has been achieved over recent years, list some questions that still need to be addressed and outline some possible directions for basic research objectives to achieve further product improvement and understanding.

FLUOROCARBON EMULSIONS FOR INTRAVASCULAR USE THE KEY ADVANCES

The carrier

Fluorocarbons do not mimic hemoglobin in any way. They are synthetic, infinitely less complex and fundamentally different in composition, structure and properties. Table II is a representative list of the kind of attributes that make some fluorocarbons attractive for use in biomedical applications.

The key selection criteria for fluorocarbons intended for intravascular use are good definition and purity, large oxygen dissolving capacity, fast excretion, absence of clinically significant side-effects and large-scale industrial availability [2,4,21]. Linear compounds are advantageous compared to cyclic ones in terms both of definition and of oxygen dissolving capacity [6]. Among the fluorocarbons investigated, these criteria appear to be met by perfluorodecalin (FDC), perfluorooctyl bromide (perflubron), and bis(perfluorobutyl)ethene (F-44E). Some other, code-named preparations have also been proposed, but their identity, structure and stage of development have not been disclosed [22]. Perflubron stands

out as it provides both more stable emulsions and faster excretion rate than the other reported fluorocarbons [1-3].

Our knowledge of fluorocarbon excretion rate *vs* structure relationships has also improved greatly. The excretion rate of "true," unsubstituted fluorocarbons decreases exponentially when molecular weight increases, independently of the presence of cycles, branches or heteroatoms in their molecular structure. It was, however, also recognized that excretion rates, as well as other properties, can be modulated to a certain extent by introducing lipophilic terminations such as a bromine atom or a linear, branched or cyclic hydrocarbon residue [23,24].

The impact of the fluorocarbon's lipophilicity on its excretion rate has been confirmed. Lipophilicity facilitates the fluorocarbon's transfer by lipid carriers in the blood from the RES cells to the lungs, where it is excreted through the expired air. Perflubron's lipophilicity, reflected by its low critical solution temperature in hexane [23,24], thus provides unusually short organ retention time.

Some additional features such as positive spreading coefficient or radiopacity can be built into the fluorocarbon molecule to meet specific applications. A positive spreading coefficient allows the liquid to spread spontaneously on an aqueous phase. Radiopacity allows for the visualization of the product in the organism using x-rays.

Perflubron is again particularly valuable in this respect because, in addition to the attributes of typical linear fluorocarbons of similar molecular weight, it has both a positive spreading coefficient and radiopacity.

New fluorocarbons and highly fluorinated materials continue to be developed to fit specific uses [25]. Perfluoroalkylated amphiphiles led, for example, to stable liposomes with modified membrane permeability [26]. Improved radiopacity has been achieved by introducing an iodine rather than a bromine atom in the structure [27]. It was found that the internal location of the iodine atom greatly reduces the chemical reactivity of the compounds compared both to fluorocarbons with terminal iodine atoms and to iodinated hydrocarbons.

The emulsions

In many applications, fluorocarbons are administered in the form of emulsions. These emulsions consist of a dispersion of small fluorocarbon droplets,

TABLE II : Fluorocarbon emulsions are different

ATTRIBUTES OF THE FLUOROCARBON

- *DISSOLVED O_2 - READILY AVAILABLE*
  HIGH EXTRACTION RATIO
- *LINEAR O_2 vs pO_2 UPTAKE - NO SATURATION*
- *NO CHELATION OF CO, NO*
- *O_2 - DISSOLUTION ↗ WHEN TEMPERATURE ↘*
- *RESPIRATORY EXCRETION*

CHARACTERISTICS OF THEIR OWN

- SMALL PARTICLE SIZES/RBC (0.2 vs 7 μm)
- NUMEROUS PARTICLES - DENSE DISTRIBUTION
  FACILITATE THE DIFFUSION OF OXYGEN
- MECHANICAL RESISTANCE (pumps, filters)
- FOREIGN PARTICLES - RES CLEARANCE
  MACROPHAGE ACTIVATION - SHORT I.V. PERSISTENCE
- SOME REFLECT SOUND WAVES, ETC
- LARGE SCALE MANUFACTURING ESTABLISHED

0.1-0.2 μm in average diameter, in a buffered aqueous phase. Each droplet is coated by a thin film of an appropriate surfactant. Fluorocarbon emulsions are different from bulk fluorocarbons, red blood cells, or hemoglobin vesicles. They exhibit most of the properties of the fluorocarbon they contain, but they also have new properties, characteristics, benefits and complications of their own. These are more related to their physical rather than to their chemical constitution (Table II).

Egg yolk phospholipids (EYP) of the same type as those used in lipid emulsions for parenteral nutrition are now generally employed as the basic emulsifier instead of Pluronic F-68, a poloxamer-type synthetic surfactant used in Fluosol, which is believed to cause complement activation [28].

Substantial progress has been achieved where emulsion stability is concerned [29]. The early fluorocarbon emulsions, exemplified by Fluosol, must be shipped and stored in the frozen state. As a consequence these emulsions must be thawed and reconstituted by the admixing of annex solutions prior to administration. The

inconvenience which results from this situation obviously limits the use of such products.

Second-generation, EYP based products are heat sterilized under standard conditions, ready for use, and can be stored unfrozen under standard refrigerator temperatures for at least a year. The use of fluorocarbons with a lipophilic extremity, such as perfluorooctyl bromide, results in enhanced stability. Enhanced lipophilic character leads to improved cohesion between the fluorocarbon droplet and the fatty acid chains of the EYP film that coats it, resulting in improved emulsion stability.

O_2-carrying capacity

The demand for emulsions with higher fluorocarbon content and hence a higher oxygen carrying capacity per dose volume, has been met [1-4,30]. For example, 90% w/v (47% by volume) concentrated, fluid perflubron emulsions have been developed which dissolve *ca* 25 vol. % of oxygen when pO_2 = 760 mm Hg [31]. For a given injected dose such products carry *ca* four times more oxygen than the rather dilute (20% w/v, i.e. 10% by volume) first-generation products (Figure 1), and thereby allow the administration of a given O_2-carrying capacity in a much lower volume. In addition, concentrated emulsions can always be diluted, for example by the simultaneous administration of a colloid or crystalloid preparation, which allows further flexibility in the treatment procedure.

The oxygen carrying capacity of fluorocarbon emulsions has also been improved by selecting linear fluorocarbons such as perflubron or F-44E, rather than cyclic ones such as FDC. For comparable molecular weights and/or excretion rates, the former have a *ca* 20% larger oxygen dissolving capacity [6].

Concentrated, small droplet size emulsions, with their larger fluorocarbon droplet population, not only increase the amount of oxygen that can be delivered to tissues by convection, but also facilitate its diffusion by reducing the distance between oxygen reservoirs in the vessels (red blood cells and fluorocarbon droplets) and the tissues [32]. The importance, for most intravascular applications, of keeping particle sizes small and particle size distributions narrow, has been confirmed. Low droplet sizes mean a large number of particles for a given dose which, for a given formulation, results in a longer intravascular persistence and lower side-effects.

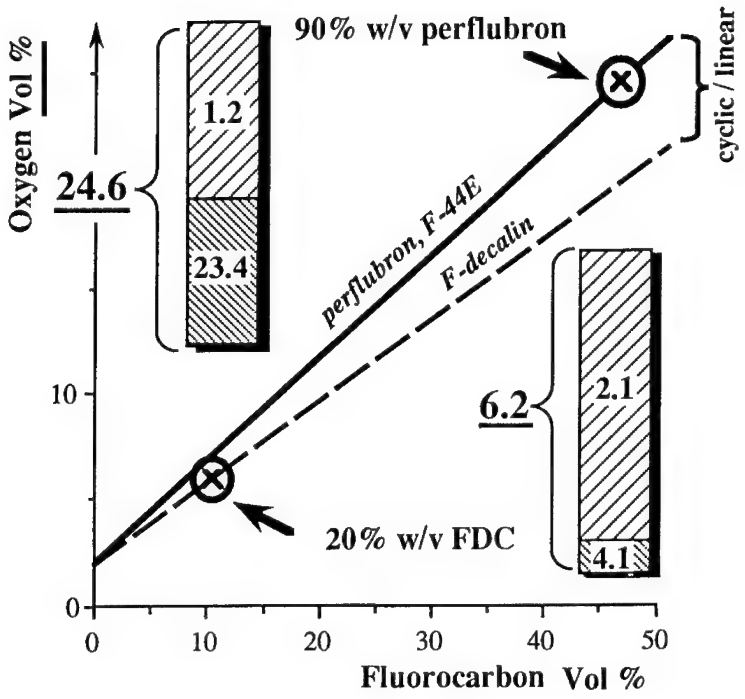

FIGURE 1: Oxygen-solubility of fluorocarbon emulsions as a function of fluorocarbon content. Strongly and lightly shaded boxes respectively represent the vol % of O_2 carried by the fluorocarbon and plasma phases in each of the emulsions represented. Note also the benefit of using linear fluorocarbons (perflubron, F-44E) rather than cyclic ones (F-decalin).

Manufacturability

Large scale pharmaceutical emulsion technology has been in use for over 20 years. Fluorocarbon emulsion technology is not much different and is now also well established. It allows the preparation and heat sterilization of small-particle-size emulsions in large amounts. Scale up for some of the newer, concentrated emulsions has already gone from lab scale research through development research, cGMP pilot and clinical size batches, to commercial scale without apparently encountering any problem specific to fluorocarbon emulsions.

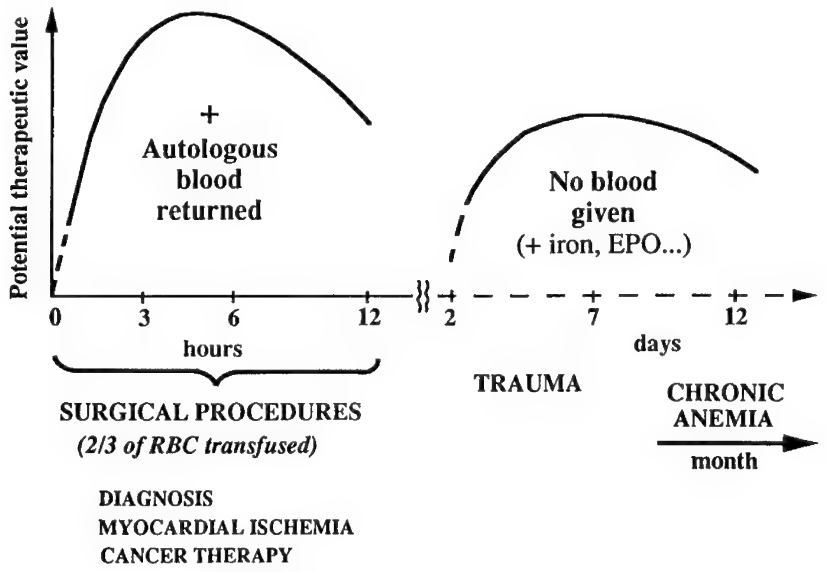

FIGURE 2: The potential therapeutic value of prolonging intravascular persistence.

Intravascular persistence

Intravascular persistence is the point on which the least progress has been made. The half-lives in the circulation of the presently-developed oxygen carriers are dose-dependent and typically in the 4 to 12 h range. The question of what therapeutic benefit can be expected from prolonging *i.v.* persistence must, however, be assessed. The present emulsions are effectual in most perioperative applications (which represent about 2/3 of the red blood cells transfused), as contrast agents for diagnosis, for treating myocardial ischemia, or when used as adjuncts in cancer radiotherapy. They will probably be of little help if the objective is to avoid homologous blood transfusion, for example, in trauma.

It must be realized that "simply" doubling the i.v. persistence of the present emulsion will not achieve this objective. To be valuable, the i.v. half-life prolongation should reach a week or longer. The treatment of chronic anemia and matching the half-life of perfused red blood cells are even further away (Figure 2). Prolonging i.v. persistence may in fact be more important because it is normally

related to lower side-effects (through depressed recognition) than for improved efficacy (unless of course it were increased by an order of magnitude).

Particle recognition and side-effects

Particulate matter, whether fluorocarbon emulsion droplets, encapsulated hemoglobin or vesicles when injected into the vasculature, rapidly undergo opsonization, i.e. adsorption of plasma proteins on their surface. Subsequent denaturation of these proteins promotes recognition and phagocytosis of the particles by the macrophage system as a normal response of the organism. Transcient activation of macrophages results and is accompanied by a chain of predictable, transient events involving the release of various substances, including prostaglandins and thromboxane of the arachidonic acid cascade, and cytokines. Substantial progress has been achieved in the understanding of the mechanism of these effects [33].

The side-effect profile induced by these substances includes a short lived (minutes) immediate response, characterized by occasional skin flushing and lower back pain, and a delayed (2-12 hours) response consisting of flu-like symptoms with febrile reactions, headaches or nausea [13,33,34]. All these effects resolve spontaneously within 12-24 hours. The extent of these side-effects is clearly dependent on some characteristics of the emulsions, including particle size, nature of the surfactant coating and processing conditions.

The delayed reactions can be controlled by corticosteroid or cyclooxygenase inhibitor prophylaxis [33].

Emulsions of some fluorocarbons, when administered in high doses to some animal species, were found to cause a phenomenon which was initially referred to as "pulmonary hyperinflation," characterized by failure of lungs to collapse at autopsy [33,35]. Rabbits, pigs and monkeys are particularly sensitive to this phenomenon. It is particularly pronounced with perfluorodecalin and with Fluosol, and present to a lesser extent with some perflubron emulsions. In the latter case, reversal is complete within 3 weeks for a 2.7 g/kg dose of fluorocarbon in rabbits. There appears to be no histopathological effect on lung tissue. Decrease in arterial pO_2 has, however, been measured in monkeys treated with large doses of certain emulsions. There are no published reports of this side effect in humans. Its

TABLE III : 1st generation vs 2 nd generation fluorocarbon emulsions : the progress

- ● BETTER DEFINED, PURE FLUOROCARBONS
- ● IMPROVED KNOWLEDGE OF FLUOROCARBONS & FLUOROCARBON EMULSIONS *ex-vivo* and *in-vivo*
- ● **FASTER EXCRETION**
- ● **BETTER ACCEPTED SURFACTANTS** (Pluronic ⟶ **egg yolk phospholipids**)
- ● **HIGHER O_2-CARRYING CAPACITY** (**linear** fluorocarbons, **fluid concentrated emulsions**)
- ● **PROLONGED SHELF STABILITY**
- ● **READY FOR USE** & USER-FRIENDLY
- ◒ **CLINICAL EVALUATION IN PROGRESS** (hemodilution, cancer, blood pool imaging, lymph node imaging)
- ◒ LIMITED SIDE-EFFECTS (+ prophylaxy)
- ◒ HIGHER VERSATILITY/ADAPTATION
- ○ I.V. PERSISTENCE : **NO PROGRESS**

mechanism has been proposed to consist of the trapping of gas bubbles in the alveoli, subsequently to gas osmosis through pulmonary surfactant-liquid bridges in the presence of fluorocarbon [36]. Pulmonary gas trapping is highly species dependant and is not expected in humans because human anatomy and physiology most closely resembles that of insensitive species [33]. Whether it has any clinical consequence in man remains to be determined. This gas-trapping phenomenon is minimized with fluorocarbons or mixtures of fluorocarbons of appropriate vapor pressure and lipophilic character.

Table III summarizes the progress that has been achieved in recent years in terms of injectable fluorocarbon emulsions.

FURTHER PROGRESS

Long-Term Room-Temperature Storage

Several effective solutions have recently been engineered which allow long-term room-temperature storage of fluorocarbon emulsions [29]. They usually

operate by counteracting molecular diffusion (a droplet size-increase mechanism, now well identified, by which individual fluorocarbon molecules leave the smaller droplets to join larger ones). This phenomenon can be hindered by adding a small amount of a high molecular weight compound, which decreases the solubility of the fluorocarbon in the aqueous phase. This solution, which has been known for some time [37] but which was unpractical because of the prolonged organ retention of such heavy fluorocarbons, has recently been made acceptable by using faster excreted, lipophilic, high molecular weight fluorocarbons as additives [24]. Perfluorodecyl bromide, a higher homologue of perflubron, is such a compound; its RES organ half-life is only of *ca* 25 days compared to 65 days for perfluorotripropylamine (one of the two fluorocarbons in Fluosol), and its stabilizing effect is much higher. Fluorocarbon emulsions can also be stabilized by reducing the interfacial tension between the fluorocarbon and water phases, i.e. by employing fluorinated surfactants. The cohesion between the fluorocarbon and the surfactant film has been improved by using mixed fluorocarbon-hydrocarbon compounds which act as molecular dowels at the interface and provide dramatic stabilization. Finally, solutions involving the addition of triglycerides have also been proposed [29].

Particle recognition and prolonged intravascular persistence

There is no doubt that further efforts are needed to prolong and control the persistence of particulates in the circulation. This is an important challenge which concerns injectable preparations containing particulates in general, whether fluorocarbon droplets or hemoglobin vesicles or liposomal preparations for drug delivery.

Injected particulates are destined to be cleared from the circulation by the RES system. Their recognition by the RES cells, and the reactions they may trigger in the organism depend on the size of these particles and even more so on the composition and structure of the external membrane that surrounds them. Particle sizes cannot be reduced indefinitely. Average particle sizes of about 0.1 μm after sterilization and the initial equilibration (or annealing) period, as well as narrow size distributions are feasible with the established technology. Smaller sizes, as in microemulsions, require significantly larger amounts of surfactants and raise new problems of their own [4].

Most of our knowledge on improving the "stealthiness" of injected particles comes from work on liposomes developed for drug delivery [38]. Conditions for prolonged intravascular persistence of liposomes were, until recently, thought to include: i) absence of net surface charge; ii) high lipid bilayer rigidity (as induced, for example, by cholesterol) and iii) high surface hydrophilicity.

Things, most often, are more complex than initially believed. Strong surface hydrophilicity definitely appears to be needed and some block polymers (poloxamers, poloxamines) are particularly suitable for providing a steric, hydrophilic barrier to suppress opsonization. But it has now also been recognized that any hydrophilic surface will not do. Attention has been drawn to the importance of steric aspects and of surface mobility. Membrane rigidity is no longer considered essential, and the presence of net charges is not necessarily contraindicated either (provided, however, they are somewhat hidden within the surfactant film). It is the presence of a sufficiently thick hydrophilic steric barrier and its mobility which are throught to best prevent the adsorption of plasma protein and subsequent uptake of particles by the RES [38].

These results suggest obvious leads to be explored for oxygen carrying particulates.

PROSPECTS

There is no doubt that long-term room-temperature stable emulsions will soon become commercially available. They will be ready for use and their concentration may depend on the application. A few basic research objectives for further product understanding and improvement can be outlined. We still need to improve our knowledge of what injected particles, whether submicronic emulsion droplets, capsules, liposomes or vesicles, do to our physiology. Prolonging and controlling the fluorocarbons' intravascular persistence and subsequent biodistribution are certainly objectives we want to accomplish.

Therefore, we must better understand the mechanism of opsonization, particle recognition, phagocytosis and the events they trigger. We certainly wish to know more about the relationships that exist between these events and the particles' characteristics, especially their surface, interfacial film or membrane structure. This should allow us to further manipulate and optimize the preparations' behavior

according to the projected use. It would also orient the synthesis and evaluation of new, more appropriate membrane components and membrane modifiers, reveal new behaviors, new synergies, etc. To be successful, such a program requires permanent and close interaction between organic chemists, colloid chemists, physiologists, pharmacologists, physicians and investigators of many other disciplines. One can predict that there will be new findings, new complications, new solutions and further applications to assess as we advance.

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OXYGEN SOLUBILITY, RHEOLOGY AND HEMODYNAMICS OF PERFLUOROCARBON EMULSION BLOOD SUBSTITUTES

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ABSTRACT

Perfluorocarbon-based blood substitute emulsions have been under development for more than a quarter century. The first generation emulsions have provided confirmation that the physical principals of high gas solubility and low viscosity can effectively support organ and organism respiration and metabolism. Clinical trials led the US FDA in 1990 to be the first to approve a 20 w/v % perfluocarbon emulsion for human use as coronary angioplasty adjuvant therapy. Hemodynamic responses to hemodilution with intravascular perfluorocarbon emulsions have varied with species and the mechanisms for adverse reactions are better understood now as second generation emulsions containing up to 100 w/v % perfluorocarbon are under development as blood substitutes, imaging agents, and for other therapeutic applications. This report describes the evolution of perfluorocarbon emulsions as blood substitutes by emphasizing oxygen solubility, rheology and hemodynamic aspects of the emulsions as they have been applied in experimental laboratory animal and human clinical settings.

Origins of Perfluorocarbon-based Blood Substitute Emulsions

Perfluorocarbons as oxygen carriers were first proposed in 1966, when Clark and Gollan [1] at the University of Cincinnati demonstrated the capacity of

perfluorocarbon liquids to support life in animals by liquid breathing. The features upon which perfluorocarbon-based blood substitute emulsions were originally and continue to be promoted are high oxygen solubility of perfluorcarbons and small particle size and low viscosity of perfluorocarbon emulsions. Gollan and Clark [2] first reported in their original paper that an isolated beating rat heart could be supported with an oxygenated mixture of 'FX-80' (perfluorobutyltetrahydrofuran and its isomers, also FC-80 or FC-75) and diluted blood. Within a year of these remarkable demonstrations, Sloviter & Kamimoto [3] at the University of Pennsylvania had prepared the first emulsion containing 40 w/v % 'FX-80' by ultrasonicating it with Krebs-Ringer bicarbonate buffer solution containing 8 per cent bovine serum albumin. They used this emulsion to perfuse the isolated rat brain and were able to maintain its electrical activity and metabolism as effectively as could perfusates containing erythrocytes.

Geyer et al. [4] at the Harvard School of Public Health took this lead and formulated a new blood substitute from perfluorotributylamine (FC-47 or FC-43), Pluronic polyethers, and physiological salts for total blood exchange in the rat. Animals with less than 1 per cent hematocrits survived up to eight hours. This early emulsion had particles as large as 1.0 to 1.5 μm with viscosities of 4.5 centipoise (cP), near that of normal rat blood. This emulsion of Geyer et al. which contained 24 w/v % perfluoro-tributylamine was the forerunner of Oxypherol™-ET (Green Cross Corp., Japan) [5].

Oxygen and Other Gas Solubility in Perfluorocarbons

Oxygen solubility of perfluorocarbons varies inversely with temperature as does oxygen solubility of water. The amount of oxygen dissolved in any perfluorocarbon increases linearly with oxygen partial pressure. This linearity continues into the hyperbaric range. Oxygen solubility in perfluorocarbons liquids exceeds considerably that of water. Even emulsions with low perfluorocarbon content (20 w/v %) can dissolve 20 times the oxygen of water. The solubility of carbon dioxide and nitrogen in perfluorocarbon fluids exceeds that of oxygen by 2 to 4 times while that of nitrogen is somewhat less than that of oxygen [6]. The solubility of oxygen in perfluorotributylamine (FC-43 / FC-47) is 38.4 mL O_2 / 100 mL fluid, in perfluorotetrahydrofuran and its isomers (FX-80 / FC-80 / FC-75) is

52.2, in perfluorodecalin (PP5) is 40.3, and in perfluoroocty lbromide (perflubron) is 52.7 if equilibrated against 1 atmosphere of oxygen gas at 25ºC [7]. Calculation or measurement of oxygen solubility in perfluorocarbon emulsions at 37ºC has yielded values of 0.79 and 0.90 mL O_2 per deciliter of FC-43 emulsion (Oxypherol™ET) at 100 torr pO_2 [5,8]. Tremper et al. [9] determined that Fluosol-DA 20% emulsion (Green Cross Corp., Japan) holds 0.6 mL O_2 per deciliter of emulsion at 37ºC per 100 torr pO_2 with 0.4 ml O_2 and 0.2 mL O_2 attributable to perfluorodecalin and perfluorotripropylamine, respectively. Oxygen solubility in perflubron emulsions of 100 w/v % (Oxygent™, Alliance Pharmaceutical Corp.) is about 3.5 mL O_2 per deciliter emulsion at an oxygen partial pressure of 100 torr.

Viscosity and Hemorheology

In vitro viscosity of perfluorocarbon emulsions is performed typically on a cone-plate viscometer, pressure cell filtration apparatus, or capillary viscometer [10,11]. Riess et al. [12] and Ni et al. [13] reported recently the viscosity of second generation perfluorocarbon emulsions as nearly flat over a wide range of shear rates for emulsions with less than 90 w/v % perflubron (perfluorooctyl bromide, PFOB). These concentrated perfluorocarbon emulsions are non-Newtonian fluids. Emulsions containing less than 60 w/v % perflubron had viscosities measured on a Bohlin CS (controlled-stress) rheometer with C25 or DG measuring geometry below that of whole human blood [13]. Extrapolations to capillary shear rates revealed 30 w/v% perflubron emulsions with viscosity of 2 centipoise (cP), while 60 w/v % emulsions were 3.2 cP.

In vivo viscometry is more difficult as the potential for dynamic biological responses to play an interactive role in blood vessel diameter is always possible. Isolated organs, particularly hearts of rats, rabbits and guinea pigs have been perfused with perfluorocarbon emulsions of varying compositions. In all cases the coronary perfusate flow rate at constant perfusion pressure, heart rate and ventricular work load is significantly lower with perfluorocarbon emulsion perfusates than with physiological salt solutions [11,14-18]. Blood with an hematocrit of 40 to 50% has a viscosity of about 5 cP at physiological flow rates observed in capillaries. Oxypherol™ET (20 w/v % FC-43) has a viscosity of 1.3

to 1.8 cP at 37°C depending upon the emulsion oncotic agent [11]. Viscosity is increased to 2.7 cP by doubling the FC-43 content in the emulsion. When the change in coronary vascular resistance and the viscosity difference between physiological salt solution and fluorocarbon emulsion perfusates are considered using the geometric resistance factor approach of Chemnitius et al. [19] no net rheologic differences can be seen between the two perfusates in the isolated working rabbit heart. The high viscosity of sickled erythrocytes can be reduced by exposure to oxygenated fluorocarbon emulsion [10]. Viscosity becomes reduced as both hemoglobin S polymerization is decreased and erythrocyte deformability is increased. This translates into improved microvascular flow and a reduction in estimated vascular resistance by 80 per cent.

Blood Residence Times

The duration of the oxygen transport supplementation provided by perfluorocarbon emulsions administered intravascularly is considered to be relatively short. Blood residence half-life is dose dependent and species dependent. Sloviter et al.[20] found that FX-80 reached 50% of initial blood levels after 3 days in mice while FC-43 had dropped to 20% over the same time. Yokayama et al. [21] observed in rabbits' blood clearance half-times of 40 hours for perfluorodecalin / egg yolk phospholipid emulsion and 52 hours for Oxypherol™ET (FC-43) emulsion. In studies where human patients with severe anemia received 20 to 40 mL Fluosol-DA™ / kg body weight, both Tremper et al. [9] and Gould et al. [22] report blood persistence half-times of 24 hours. In the summary of nearly 200 human cases in Japan, Mitsuno et al. [23] found that blood retention was dose dependent as 50% persistence rose from 6 hours to about 24 hours when the dose was increased from 500 to 1000 mL of Fluosol-DA™(20%). The levels of perflubron measured by 19F NMR spectroscopy in blood of beagles administered Oxygent™ intravenously at 6 mL emulsion / kg body weight had fallen to 50% of the original blood level in 48 hours [24]. Blood persistence time is known to relate to dose administered and properties of the specific fluorochemical emulsion.

Preservation of Ischemic Tissue and Tissue Oxygenation

A major concept predicated upon the small particle size, low viscosity and high oxygen content of perfluorocarbon emulsions has been that administration should protect tissue from hypoxia and prevent irreversible tissue damage. Emulsions

have been shown to not only support the metabolism and electrical activity of the isolated, perfused brain [3] but also to reduce the injury associated with obstruction of a major cerebral artery [25]. Brain oxygenation improves with fluorocarbon emulsions added to the blood out of proportion to the amount of oxygen dissolved by the fluorocarbon [26]. The suggestion that fluorocarbon emulsions facilitate capillary to tissue oxygen transfer has also been proposed from retinal oxygenation studies by Braun et al. [27].

In the heart, where many tissue protection studies have been conducted [28-31], fluorocarbon emulsions improve blood flow distal to stenosed coronary arteries [32], increase oxygenation in myocardial regions dependent upon flow through pre-existing small collateral arteries [33], and prevent regional myocardial contractility failure when used as a perfusate during coronary angioplasty procedures in humans [34]. This latter demonstration led in 1990 to the US Food and Drug Administration approval of Fluosol-DA™ for human use.

In a variety of other organs, species and conditions, fluorocarbon emulsions have been demonstrated to improve tissue oxygenation. In severely anemic humans given 20 mL Fluosol-DA per kg body weight, Tremper et al. [9] used a cutaneous oxygen electrode to track systemic oxygenation as well as obtain an indication of peripheral circulatory oxygenation. Skin pO_2 rose markedly when the emulsion was present. Tissue oxygen partial pressure has also been shown to increase after hemodilution with perfluorocarbon emulsions in visceral organs [35-38] and skeletal muscle [39] of laboratory animals. No parallel measures of blood flow have been made in these tissues to assess hemodynamic factors that may contribute to the observed improvements in tissue oxygenation.

Hemodynamics in Laboratory Animals and Humans

The effects of perfluorcarbon emulsion hemodilution on organ blood flow and the distribution of cardiac output has recieved little direct attention. The major organ to be studied with respect to blood flow and flow distribution is the heart. Studies in the isolated, perfused mammalian heart have demonstrated the ability of fluorocarbon emulsions to support better oxygenation, metabolism and contractile function at lower perfusate flows than obtained at the same perfusion pressure with physiological salt solutions [11, 14-19]. Indirect evaluations of blood flow

changes using tissue oxygenation have been made in the retina, brain, liver, and skin as noted above. Kidney functions have been evaluated after a single dose of one perfluorocarbon emulsion formulation and found to be essentially normal with the observed changes attributable to the volume load produced by addition of the emulsion to the blood [40].

Earlier human studies [9,32] showed small decreases in heart rate and increases in arterial oxygen tension without change in cardiac output or aortic blood pressure in severely anemic patients with hemoglobin levels of <4.5 g%. Pulmonary circulation variables that have been monitored include pulmonary artery diastolic and systolic blood pressures cardiac output, pulmonary vascular resistance and pulmonary capillary wedge pressure. While most studies have reported a low incidence of changes in these variables, others have noted elevations in pulmonary artery pressures [9]. Systemic circulation variables have included cardiac output, peak aortic flow, aortic diastolic and systolic blood pressures, and systemic vacular resistance. Again most studies report little change in these variables, yet profound systemic hypotension has been reported in humans[41] and reduced cardiac output have been reported in some animal models [42]. Cardiac variables most commonly reported include heart rate, left ventricular peak systolic blood pressure, first derivative of left ventricular blood pressure, left ventricle end diastolic blood pressure, myocardial shortening fraction and coronary blood flow. Indices of myocardial oxygenation status such as lactate and pyruvate balance, oxygen consumption, creatine kinase release, and myocardial ATP concentrations have also been examined. Most myocardial indices have been reported to be little affected by intravascular administration of fluorocarbon emulsions. In fact, the contractile dysfunction associated with coronary blood flow reduction during angioplasty procedures is prevented by local infusion of oxygenated fluorochemical emulsion [34].

Cardiovascular reactions, mechanisms and prophylaxis and treatment

Small volumes of intravascular perfluorocarbon emulsion have been shown to produce profound hemodynamic effects in laboratory animals and in human volunteers and patients [9, 43-45]. This response has been noted with some but not all emulsions and in not all species nor in all individual subjects within a given species. When observed, the adverse response may include but not be limited to

pulmonary hypertension, chest "tightness", systemic hypotension, and elevated central venous pressure. These responses can also occur with the intravascular administration of non-perfluorocarbon emulsions. This sequelae of events remains of such concern that a "test" dose of 0.5 to 1.0 mL of the perfluorocarbon blood substitute emulsion is administered routinely as a screening procedure before substantial volumes are added to the blood pool. Prophylactic measures including cortisol, indomethacin, aspirin, and dexamethasone have been advocated and demonstrated to be effective in blunting or averting "adverse" hemodynamic responses to initial and subsequent doses of perfluorocarbon emulsions [46,47]. Species sensitivity to perfluorocarbon emulsions has been reported, with swine the most sensitive, dogs variably sensitive, and non-human primates least sensitive [42, 44, 45]. Humans who receive fluorocarbon emulsions can experience the full range of hemodynamic "reactions" and caution is still required even when premedications designed to avert adverse responses are given.

The Future

Religious objectors to blood transfusion (e.g.,Jehovah's Witnesses) remain the primary recipients of large volumes of perfluorocarbon emulsions as oxygen-carrying blood substitutes. However, with the emerging opportunity for use of stable, highly concentrated perfluorocarbon emulsions as adjuvant therapy in cancer diagnosis and treatment and as vascular imaging agents, significant expansion of the recipient patient population is predicted. Despite relatively small volumes of emulsion necessary for some human applications, it remains important to establish the systemic hemodynamic consequences of fluorocarbon emulsion addition to depleted blood volume and total hemoglobin so as to better understand the potential for them as effective resuscitant and elective hemodiluents. With proper understanding of laboratory species' hemodynamic sensitivities to emulsions, including those composed with fluorocarbons, and inclusion of appropriate placebo controls in experiemtnal designs, this information should be available by the end of the decade.

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THE POTENTIAL ROLE OF OXYGEN-CARRYING PRODUCTS IN AUTOLOGOUS BLOOD TRANSFUSION PROTOCOLS

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ABSTRACT

For surgical patients transfusion of autologous blood (AB) is the most useful of measures to reduce patient's exposure to homologous blood (HB). In our Institute an autotransfusion program was started in 1982 utilizing all the autotransfusion techniques currently available. The integrated use of the techniques offered to the majority of the patients the possibility of receiving AB (98% of the elective surgery patients) and a consistent conservation of HB has been achieved (60-70%). However 42% are still exposed to some HB.

Critical parameters that render the patients unable to fullfill the anticipated transfusion needs with the current AB transfusion techniques are: the patient's ability to predonate sufficient AB prior to surgery and the amount of blood transfused intraoperatively that in turn depends on different "transfusion trigger". In our Institute over 50 % of all the blood units are transfused the day of operation (60% being AB, 40% HB) and 50% postoperatively (only 33% being AB).

For this reason, a clinical application for the oxygen-carrying products can be the replacement of the blood lost during, or immediately after the operation permitting the surgeon to operate safely at a lower Hct levels, thereby delaying the transfusion of blood and saving the AB obtained.

INTRODUCTION

Until recently blood transfusion was considered an harmless adjunct to surgery; however the knowledge that acquired immunodeficiency syndrome (AIDS) could be

transmitted with blood focused the attention on the risks of blood transfusion, reinforcing the long established reasons for avoiding the transfusions of homologous blood: transmission of infectious diseases, transfusion reactions, and immunomodulation leading to increased mortality from infection and earlier recurrences of cancer [1,2,3].

Through a wider cooperation between surgeons and transfusionists, in many centers a more rational use of blood and blood products and a reduction of the number of nnecessary transfusions has been achieved [4]. As any homologous blood transfusion should be avoided when safer alternatives are available a variety of alternatives to homologous blood transfusion have been proposed: transfusion of autologous blood, use of solutions to support oxygen transport (modified hemoglobin solution or perfluorochemical emulsions) and human recombinant erythropoietin (r-HuEPO). At present only autotransfusion is considered an established alternative to homologous blood transfusion and for surgical patients autologous blood transfusion is potentially the most useful of measures to reduce exposure to homologous blood [5,6,7,8,9,10,11]. Other alternatives, although promising, are still considered experimental. Modified hemoglobin solutions and perfluorochemical emulsions are under study as "artificial blood", however although good results have been obtained in animals, their clinical application is still not established [12,13,14]. Recently r-HuEPO therapy has been proposed to stimulate erythropoiesis in the pre- and post-operative periods in order to reduce the need for transfusion [15,16,17,18,19].

Interest in autologous transfusion continues to grow due to the media-driven, patient's demand, hospital and physicians concern about litigation. Though the programs are enthusiastically promoted by physicians, patients and politicians through specific laws, their utilization is still limited. Several factors contribute to this lack of progress. First, it is generally difficult to transform autotransfusion trials into a systematically used procedure in a hospital organised for homologous blood transfusion. Secondly, the inertia to change professional habits and the perception that an autotransfusion program is an inconvenience remains strong because of the difficulties in organization for the surgeon, anaesthetist and blood bank. Thirdly, there are limitations related to the methodologies for obtaining

autologous blood: it is necessary to allow time before surgery to donate autologous blood, the operation must be carefully programmed, in emergencies only perioperative salvage can be used and in many cases the autotransfusion procedures cannot be used because of organizational problems.

As a successful autologous program should enrol all appropriate patients, reduce the use of homologous blood and avoid the exposure to the risks of donor blood, some authors question the efficiency of the autologous transfusion programs because in many cases autologous donors are still transfused with homologous blood. The integrated use of all the autotransfusion techniques, preoperatively (autologous blood predonation, perioperative isovolemic hemodilution) intra and post operatively (salvage of blood, washing and reinfusion) allows the enrolment of most of the surgical patients into autotransfusion programs. For patients undergoing major surgery the chance of avoiding the transfusion of homologous blood depends on beeing enrolled in an autotransfusion program, on the perisurgical blood loss and on the amount of autologous blood that can be rendered available by the integrated use of all the autotransfusion techniques.

In order to establish the specific role of each of the alternatives to homologous blood in contributing to the success of the program, the results obtained in Orthopedic Institute G.Pini, concerning the objective of including as many patients as possible in the program, of transfusing autologous blood only in order to avoid the exposure to the risks of homologous blood and conserve donor blood, have been reviewed.

ROLE OF AUTOLOGOUS PREDONATION AND PERIOPERATIVE SALVAGE

From January 1982 up to December 1992, a total of 8102 patients undergoing major orthopedic surgery were enrolled into the autotransfusion program; from these patients 28453 autologous blood units have been obtained.

During the last five years practically all the patients undergoing elective surgery received autologous blood and the results for 1992 related to enrolment, blood conservation, and exposure to homologous blood are reported in table 1.

It is shown in the table that despite the fact that 98% of elective surgery patients were enrolled and more than 75% of the blood transfused was autologous, only

TABLE I: RESULTS OBTAINED WITH THE INTEGRATED USE OF AUTOLOGOUS PREDONATION AND PERIOPERATIVE SALVAGE

	ALL PATIENTS	ELECTIVE SURGERY	NON ELECTIVE SURGERY
ENROLMENT	80 %	98 %	52 %
BLOOD CONSERVATION	63 %	75 %	27 %
EXPOSURE: NO	42 %	53 %	25 %
PARTIAL #	38 %	45 %	28 %
TOTAL	20 %	2 %	47 %

Decreased > 50 %

50% of the patients enrolled fulfilled transfusion needs with autologous blood only while employing the current autologous transfusion techniques. The following factors emerged as critical for transfusing homologous blood to autologous program patients: 1) the number of blood units actually transfused, 2) the autotransfusion techniques which could be used for the single patient and 3) the number of autologous units that the patients could predeposit in the limited period before surgery.

ROLE OF ERYTHROPOIETIN

Presurgical collection of autologous units is affected by the total circulating volume of red blood cells (that depends on body mass and hematocrit -Hct-) and by the rate of recovery of Hct after red cells are collected (which depends on the degree of stimulation of erythropoiesis during the intervals between blood collections) [20,21,22].

In patients unable to predonate the requested number of blood units, administration of r-HuEPO may be effective in correcting anemia induced by homologous blood donation and for increasing the yield of autologous blood, thus r-HuEPO has the potential to increase the number of surgical procedures which can be carried out with the sole use of autologous blood.

In a prospective and placebo controlled experiment carried out in our Institute, 50 women, undergoing total hip replacement (THR) with a baseline Hct < 40%, who received r-HuEPO in doses of either 300 U/kg or 600 U/kg twice a week for three weeks, were able to predeposit significant amounts of autologous units. Exposure to homologous blood was prevented in 84% of r-HuEPO treated patients but only in 50% of the patients in placebo group [23].

POTENTIAL ROLE OF OXYGEN CARRIERS

Another critical parameter that renders the patients unable to fulfil the anticipated transfusion needs with the current autologous blood transfusion techniques is the different amount of blood transfused intra and postoperatively which in turn depends not only on the type of surgery performed but also on different transfusion triggers.

Transfusion triggers can be easily managed in the post-operative period through the cooperation between the medical staff and the personnel of the transfusion service. In our Institute a policy has been agreed that post-operatively blood for transfusion can be released by the transfusion service only when Hct is lower than 28%. When Hct is higher, transfusion treatment must be discussed between the doctor in charge of the patient and the doctor of the transfusion service.

What is difficult to control is the appropriate use of transfusion trigger in the perioperative period (intra and immediately post-operative). We observed that 75% of all patients operated in our Institute are transfused the day of operation and that over 50% of all the blood units are transfused on that day (60% being autologous, 40% homologous). 50% of blood is then transfused in the postoperative period.

The use of blood or blood components while the patient is still bleeding should be avoided by using products that can safely bridge this fase and to only transfuse blood or components when sufficient hemostasis has been achieved. For this reason, the ideal clinical application for oxygen-carrying products, with the presently documented limitations (brief intravascular half-life, and dose limiting side effects), is to use them during surgical procedures to compensate for anemia due to either intentional blood removal (autologous predonation and/or perioperative isovolemic hemodilution) or unintentional surgical and/or traumatic blood loss. The replacement of the blood lost during, or immediately after the

operation with the oxygen-carrier drugs may permit the surgeon to operate safely at lower Hct levels, thereby delaying the use of the blood transfusion. The use of an oxygen-carrying drug in association with autotransfusion techniques and recombinant human erythropoietin could optimize the autotransfusion program in order to further decrease the need for homologous blood transfusion.

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Acute Myocardial Ischemia: Effects of Reperfusion with Arterial Blood

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ABSTRACT

Periods of severe ischemia of 15 minutes or less injure myocytes of the dog heart *reversibly* in that reperfusion of the affected tissue with arterial blood salvages all myocytes destined to die if the ischemia is not relieved. While the myocytes are ischemic, they develop numerous changes as a consequence of ischemic metabolism including depletion of ~P and accumulation of glycolytic intermediates, H^+, and the endproducts of adenine nucleotide pool degradation. With restoration of arterial flow, aerobic respiration resumes. Lactate and other intermediates are reutilized or are washed to the systemic circulation. If the period of severe ischemia is extended to 40-60 minutes, the injury becomes *irreversible.* Such myocytes cannot be salvaged by reperfusion with arterial blood and are necrotic. When reperfused, irreversibly injured myocytes develop contraction-band necrosis and accumulate calcium phosphate. Although unproved, it is possible that some myocytes, alive at the time of reperfusion, may die as a consequence of successful reperfusion. This phenomenon is termed *lethal reperfusion injury.* Sublethal forms of reperfusion injury, such as stunning, also occur.

INTRODUCTION

Many of the myocytes destined to die in areas of regional ischemia in large animal hearts survive for several hours. These living myocytes are *reversibly injured* and resume function if they are reperfused with arterial blood. Although alive, they are damaged by the ischemic process; this damage is variable in its extent and requires minutes, hours, or even days to be repaired. Eventually however, the damaged myocardium returns to the control condition. On the other hand, after prolonged ischemia, the damaged myocytes pass into a phase of *irreversible injury*. Irreversibly injured myocytes cannot be salvaged by reperfusion. In this paper, we shall summarize the pathophysiology of ischemia, the response of ischemic myocardium to reperfusion with arterial blood, and the theoretical basis of assessing the effects of therapy designed to delay or prevent ischemic myocyte death.

MATERIALS AND METHODS

Our experiments have been performed on the hearts of healthy mongrel dogs weighing 12 to 25 kg. The methods used are described in detail elsewhere [1,2] but they will be described briefly in the following paragraphs.

Under intravenous sodium pentobarbital anesthesia, using a Harvard Model 607 respirator pump and maintaining blood pH, pO_2, and pCO_2 in a physiologic range, the left chest was opened and the circumflex branch of the left coronary artery was isolated under the left atrium. An umbilical tape was placed around it and it was occluded by pulling the vessel up into a glass tube and clamping the ends of the tape. Electrocardiographic records were taken to follow the course of the ischemia. Arterial flow to the myocardium was measured by radioactive plastic microspheres. Where indicated, thioflavine S was injected 15 seconds prior to excision of the heart in order to define the zone of ischemia [3].

Metabolic Studies: At the completion of the experiment, the heart was excised quickly along the atrioventricular groove and was placed in 750 ml of ice-cold (0°C) isotonic KCl for exactly one minute. During excision and cooling, enough ischemic metabolism takes place to decrease the CP

of control tissue from 35-40 [4,5] to 9-20 [6] μmol/g dry. However, ATP remains essentially unchanged.

After cooling, transmural slabs of left ventricle from the anterior descending and circumflex beds were frozen in freon at liquid N_2 temperatures. These then were dehydrated while frozen in a Virtis lyophilizer (Model 6201-6220). When dry, ischemic and non-ischemic tissue was identified from the pattern of thioflavine S distribution and samples were counted for arterial flow. After estimating arterial flow, these samples were redried under vacuum. The remaining wet heart tissue also was counted for arterial flow.

Metabolite Assays: After trimming the endocardium and epicardium from the dry tissue, it was powdered in a mortar and pestle. 20-70 mg samples of powder were weighed and added to perchloric acid for extraction of metabolites. After centrifugation, the extracts were neutralized with a mixture of 1M K0H and 1M K_2C0_3. ATP, ADP, AMP, adenosine (ADO), inosine (INO), hypoxanthine (HX), and xanthine (X) were measured by high performance liquid chromatography [7]. ATP, CP, glucose-6-phosphate (G-6-P), glucose-1-phosphate (G-1-P), alpha glycerol phosphate (αGP), and lactate were measured by enzymatic techniques described previously [2]. Glycogen was measured in the powder by amyloglucosidase digestion followed by enzymatic measurement of glucose in order to estimate glucose liberated from glycogen [8]. Most enzymatic assays were run in duplicate; thus, results from individual tissue samples usually are the mean of duplicate analyses.

Myocardial Blood Flow: Regional myocardial blood flow was assessed with 10 ± 1 μm radioactive microspheres. Microspheres (New England Nuclear, Boston, MA) were agitated mechanically on a Vortex mixer and ultrasonically in a bath for at least 20 minutes before use. At times given in the experimental design section, two to three million spheres labeled with ^{153}Gd, ^{46}Sc, ^{113}Sn, or ^{141}Ce (3 isotopes/animal) were injected through a left atrial catheter, followed by a 15 ml of saline flush. Reference arterial blood samples were withdrawn from the femoral artery at a rate of 7.75 ml/minutes, beginning just before and continuing for 2.5 minutes after injection[9].

Infarct Sizing Studies: Area-at-Risk: To determine the anatomic boundaries of the previously ischemic and nonischemic vascular beds, two different dyes, triphenyltetrazolium chloride (TTC) (1%, phosphate dextran buffer, Sigma) and monastral blue dye (4%, phosphate dextran buffer, Sigma) were injected simultaneously at 37°C under 120-140 mm Hg pressure into the previously occluded LAD and the left main coronary artery, respectively. The heart then was fixed by immersion in a large volume of phosphate buffered formalin. The fixed hearts were cut into eight transverse slices which were weighed and their apical surfaces were photographed. The area at risk was identified and traced from an enlarged projection (magnification X 8) of the color transparency of each ventricular slice, and quantitated using a digitizing table interfaced to an IBM compatible personal computer.

Myocardial infarct size was estimated by histologic techniques. The methods required for the procedure are described in detail in reference [9].

Regional Myocardial Blood Flow: The remaining myocardium was processed for measurement of myocardial blood flow. The slices were divided into nonischemic and central ischemic regions, with the central ischemic region comprising 60-75% of the area at risk. Lateral and septal zones were excluded to avoid errors associated with measuring blood flow in samples of heterogeneous composition. The samples were subdivided further into subepicardial, midmyocardial, and subendocardial thirds. Tissue and reference blood radioactivity were measured in a Packard A5912 gamma counter, with correction for overlap of isotope spectra. Myocardial blood flow (expressed as ml per min per gram wet weight) was calculated as:

$$\frac{(\text{tissue counts})\,(\text{reference flow})}{(\text{reference counts})}.$$

Corrections were made for apparent microsphere loss if the preocclusion ischemic region/nonischemic flow ratio was less than 0.9, based on the assumption that apparent microsphere loss is a reflection of edema, inflammation, or hemorrhage causing an artifactual underestimation of blood flow and overestimation of area at risk and infarct size [10].

Statistical Methods: Experimental groups means ± standard errors of the mean are reported in the figures. Differences between means were assessed by two-tailed paired or unpaired t-tests. For group comparison of infarct sizes and ancillary parameters, dogs which did not develop severe ischemia (subendocardial collateral blood flow 0.15 ml/min/g) were excluded. However, all animals were included when comparing the relationship between infarct size and collateral blood flow. When comparing changes in hemodynamic or functional parameters over time within a given group, repeated measures analysis of variance was used. When the mean infarct size or collateral blood flow was compared among the four groups, an analysis of variance was used, with subsequent t tests to detect group differences. To test for differences in the relationship between infarct size and collateral blood flow, analysis of covariance was performed, using infarct size as the dependent variable and collateral blood flow as the independent covariate. In all analyses, a P value less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Arterial Collateral Flow: In the average canine heart, there are small arterial connections ranging from 20-200 μ in diameter between the major arterial beds serving the left ventricle. Thus, when a major artery is occluded proximally, blood flows through these connections into the occluded bed. The magnitude of this arterial collateral flow is shown in Figure 1. Note that the inner layer in all but three hearts was severely ischemic, i.e., it received flows of less than 10% of control.

Note that there is a transmural gradient of flow such that the inner layer of the heart receives the least and the outer layer the most flow. In fact, the subepicardial myocardium exhibited flows that were as high as 50% of control flow. This *transmural gradient of ischemia* is characteristic of hearts with significant collateral connections between arterial beds. The transmural gradient is caused by the reduced coronary arterial pressure found distal to the narrow connections between the arterial beds and because coronary flow to the inner layers of the heart is not continuous but occurs only during diastole. It seems likely that some flow occurs in

the subepicardial zone during systole. This may contribute to the greater collateral flow usually found in this zone.

Note that three of the 31 hearts in Figure 1 exhibit virtually no arterial flow to any layer of the heart after occlusion. In these hearts, occlusion results in transmural severe ischemia. If this ischemia is unrelieved, a transmural infarct will develop quickly and involve most of the myocardium at risk [11].

The hearts of some species, e.g., those of the pig and rabbit virtually always lack significant collateral connections. These hearts develop transmural infarcts, i.e., infarcts that involve 95% or more of myocardium-at-risk. In these hearts, cell death occurs quickly; cell death is complete after less than 90 minutes of ischemia [11].

In order to study the processes leading to cell death in ischemic tissue, it is important to use tissue that is uniformly injured. Most of the metabolic studies described in the next section were performed on severely ischemic subendocardial myocardium of the dog heart.

Metabolic Effects of Regional Ischemia: Sudden occlusion of a coronary artery in a healthy dog is followed by a marked reduction in arterial flow to the myocardium supplied by that vessel. Within 8-10 seconds, the oxygen trapped in the tissue as oxyhemoglobin and oxymyoglobin is utilize [12], oxidative phosphorylation ceases and anaerobic glycolysis supervenes as the only significant source of new high energy phosphate (~P). At about the same time, electrocardiographic changes appear, contractile activity becomes inefficient and shortly thereafter ceases (Figure 2). Most of the creatine phosphate (CP) of the heart is utilized in the first few beats after the onset [4,13]. At about the same time, the myocytes begin to swell, as a consequence of the ever increasing load of osmotically active particles (osmolar load) being generated inside the myocytes by ischemic metabolism [14]. Lactate, α-glycerol phosphate (αGP), inorganic phosphate (Pi) and H^+ all increase rapidly. The magnitude of the increases in several representative metabolites is shown in Figure 3.

The increase in the osmolar load causes the myocyte to become edematous because of the acute increase in the number of osmotically

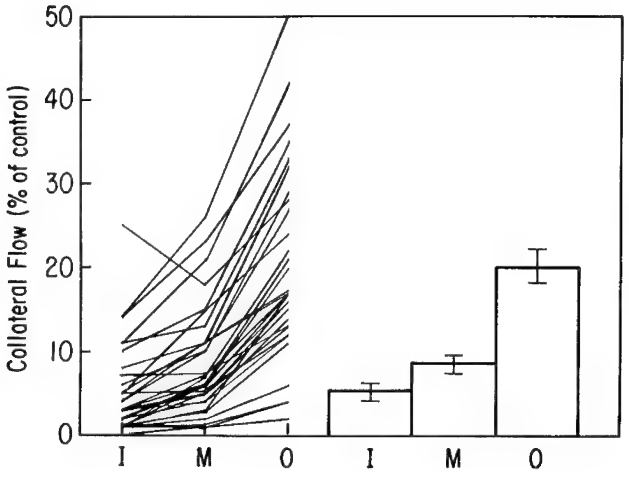


FIGURE 1. Transmural distribution of collateral flow 20 minutes after proximal circumflex artery occlusion in 31 dogs. Flow was measured with 9±1 μm microspheres before and after coronary artery occlusion. Collateral flow is expressed as percent of preocclusion flow to the same samples. Individual dogs are illustrated on the left, and the group mean ±SEM are shown on the right. I, M, and O are inner, middle, and outer thirds, respectively, of the transmural wall in the circumflex bed. There was usually a transmural flow gradient such that flow to the outer wall was greater than flow to the inner wall. Subendocardial flow almost always was depressed severely (<10%) and averaged 4.5% of control. Subepicardial flow was greater (averaged 20% of control) and much more variable than subendocardial flow. (Reprinted with permission of the publishers of Reference [36]).

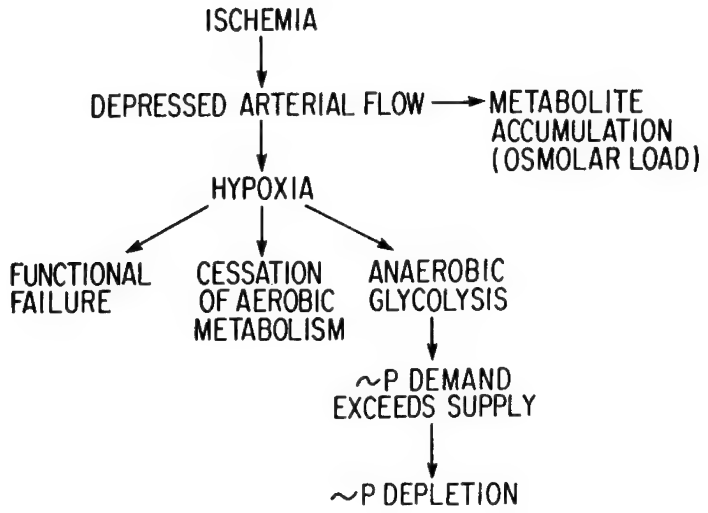


FIGURE 2. Principal consequences of the reduction in arterial flow are shown in this diagram. Metabolites are produced intracellularly by hypoxic metabolism where they accumulate (the osmolar load) and equilibrate to a variable extent with extracellular fluid. Since tissue demand for high-energy phosphate (~P) exceeds supply, the net level of adenosine triphosphate decreases until it is virtually zero in zones of low-flow ischemia. (Reprinted with permission of the publishers of Reference [6]).

active particles. The result is a volume and perhaps energy dependent export of K^+ ion from the intra- to the extracellular space [14,15]. This results in reduction in the membrane potential and contributes to the electrocardiographic changes of ischemia. However, although myocyte swelling develops quickly, swelling does not become massive; it is limited by the small volume of extracellular fluid available in severely ischemic myocardium [14].

Most of the metabolic changes that develop in the ischemic myocardium develop because the demand of the ischemic myocytes for

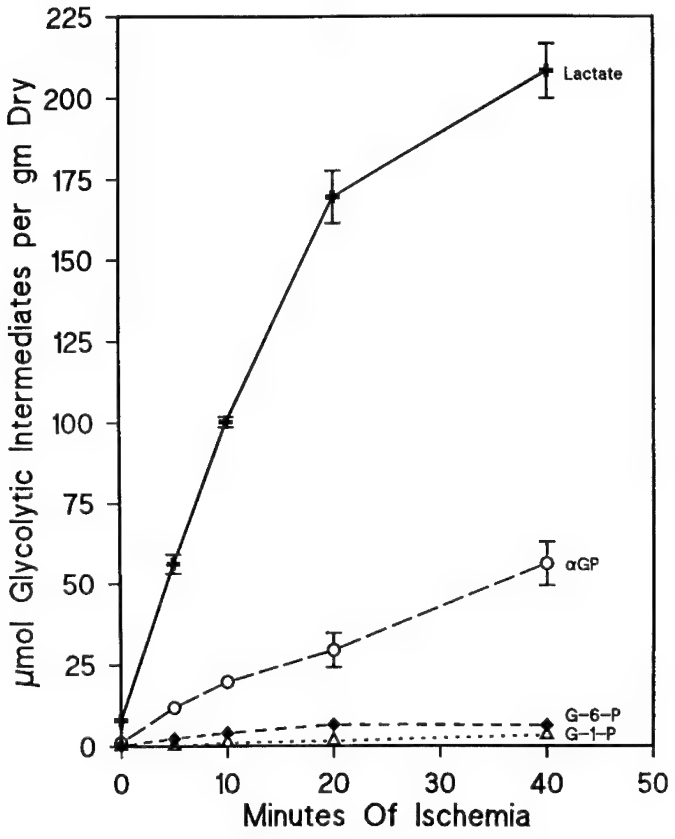


FIGURE 3. Rate of accumulation of various glycolytic intermediates (μmol glycolytic intermediates per gm dry) as a function of duration of severe ischemia (minutes). Results were obtained from groups of 4-6 hearts subjected to 5, 10, 20, and 40 minutes of in vivo ischemia. In each heart, flow to the tissue was reduced to <0.07 ml/g. The rate of glycolysis, as judged by the accumulation of lactate, slowed after 20 minutes of ischemia had passed. From other studies, it is known to stop after 40 minutes of ischemia have passed. The source of glycolytic intermediates is glycogen. At least 25 μmol and as much as 100 μmol glycogen were present in each heart studied at 40 minutes, a fact which suggests that substrate deficiency is not a factor in cessation of glycolysis in this system. αGP , α glycerol phosphate, G-6-P, glucose-6 phosphate; G-1-P, glucose-1-phosphate. (Reprinted with permission of the publishers of Reference [16]).

~P exceeds the supply [16]. The demand is due to, 1) the continued attempts of the myocytes to contract, 2) the continued electrical stimuli entering the ischemic region driving various transport ATPases, 3) the mitochondrial ATPase, and 4) and a variety of enzymatic reactions such as adenyl cyclase and fatty acid CoA synthetase that continue to act while the tissue is ischemic.

The supply of ~P available to the ischemic tissue is derived chiefly from reserve ~P and from anaerobic glycolysis. The reserve supply of ~P is found as CP, ATP, and ADP and is scanty. In dry left ventricular myocardium, only 100 μmoles ~P/g dry are available from this source [6].

Anaerobic glycolysis provides the only source of new ~P available to the myocyte. It begins about 8-15 seconds after the onset of ischemia, uses glucose-1-phosphate(G1P) from glycogen as substrate and releases 3 μmoles of ATP per μmol of G1P converted to lactate. This is much less efficient than aerobic glycolysis where 38 μmoles of ~P are produced per μmol of glucose oxidized to $C0_2$ and water. In addition, the products of anaerobic glycolysis that accumulate in the myocyte, particularly H^+ ion and lactate, inhibit glycolysis [17]. It may be beneficial for the glycolytic rate to slow because the supply of glycogen is finite, about 130-300 μmol/g dry in the dog heart. In most cases, however, tissue glycogen does not limit ischemic energy production; glycolysis ceases before the glycogen supply is exhausted (Figure 3) [18].

The ATP and lactate of the tissue serve as a measure of the supply-demand imbalance of ischemia (Figure 4). They show an inverse relationship; net myocardial ATP decreases while lactate rises. The lactate rises as soon as anaerobic glycolysis begins. The initial glycolytic rate is high but it slows 60-90 seconds after the onset of ischemia [17]. Anaerobic glycolysis ceases when the tissue lactate reaches 210-300 μmoles/g some 40 minutes after the onset. Lactate accumulates because it cannot be metabolized further in the absence of 0_2 and because there is no arterial flow to wash it to the systemic circulation.

ATP begins to decrease as soon as the CP of the myocardium is utilized, some 30-60 seconds after the onset. Net myocardial ATP

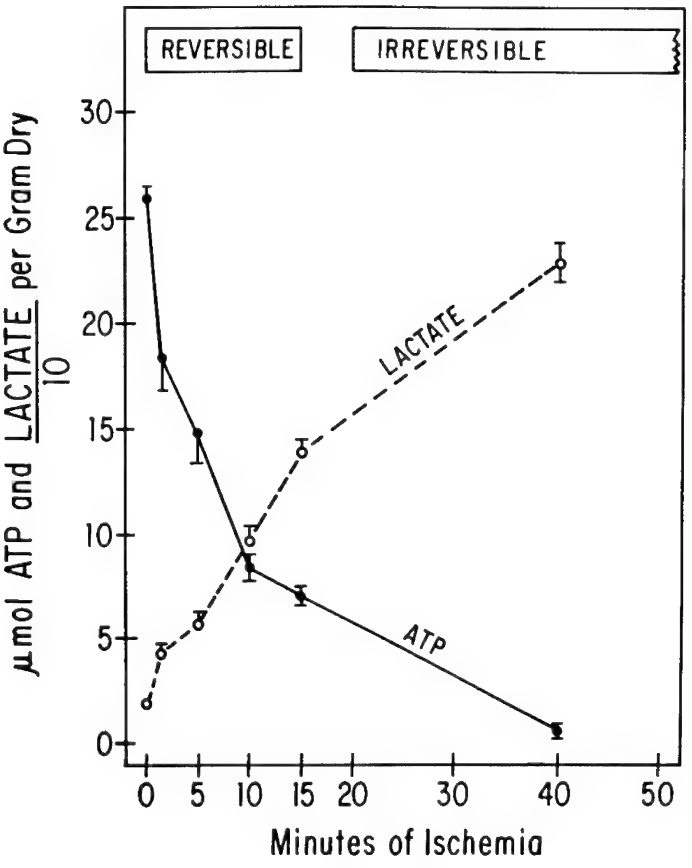
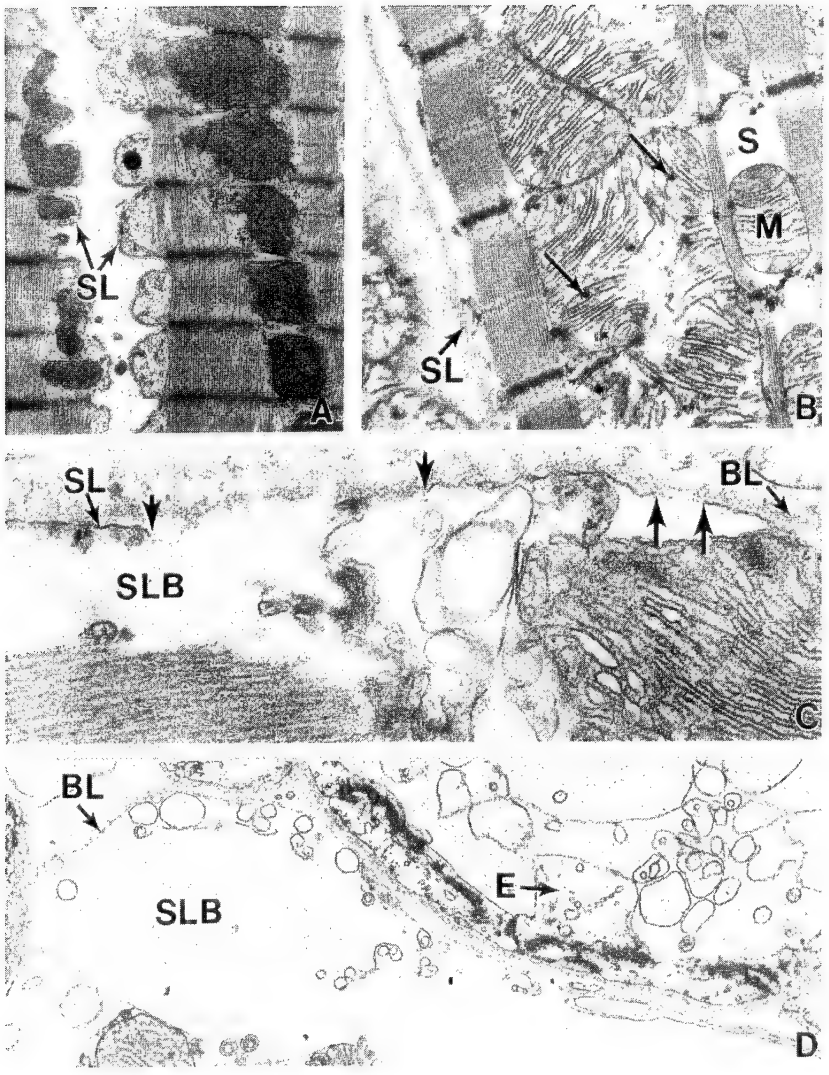


FIGURE 4. Adenosine triphosphate (ATP) depletion in μmol/g dry (•---•) versus lactate production (o---o) in low-flow regional ischemia. Results were obtained from severely ischemic subendocardial myocardium of groups of 4-6 hearts of anesthetized open-chest dogs subjected to occlusion of the circumflex branch of the left coronary artery. The tissue was frozen, dried, and analyzed after estimating arterial collateral flow with microspheres. In no case was collateral arterial flow >0.07 ml/min/g. Brackets indicate SEM. The injury is considered to be reversible during the first 15 minutes and irreversible after 20 or more minutes of ischemia. (Reprinted with permission of the publishers of Reference [37].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decreases very quickly during the first 10 minutes of ischemia; then the rate of depletion slows [6,16,19] (Figure 4). About 40 minutes of ischemia are required to exhaust myocardial ATP.

During ischemia, the adenine nucleotide pool is catabolized to the nucleosides adenosine (ADO) and inosine (INO) and the bases hypoxanthine (HX) and xanthine (X). At the end of 40 minutes of [20]

FIGURE 5. Control and irreversibly injured myocytes from canine left ventricle damaged by in vitro ischemia and fixed in glutaraldehyde buffered with 0.1 M cacodylate (approximately 600-650 millisomolar) followed by post-osmication. (a) is representative of control non-ischemic myocardium. The sarcolemma (SL) is anchored to the underlying myofibrils at the Z-bands. The sarcoplasm is compact and contains granular glycogen and mitochondria. (b) is a portion of a representative myocyte irreversibly injured by 40 minutes of permanent ischemia. The myocyte is swollen; note that the SL has detached from the markedly relaxed myofibrils and covers a bleb of edema fluid. Note the swollen mitochondria (M) containing amorphous matrix densities (arrows). The sarcoplasm (S) is clear and is increased in volume. Few glycogen granules are present. (c) is a high power view of plasmalemmal disruption after 40 minutes of in vivo ischemia. The plasmalemma is designated SL and the point of disruption is shown between the thick arrows where there is persistent basal lamina (BL) but no unit membrane. A subsarcolemmal bleb (SLB) is present where the SL is lifted off the myofibril. This tissue was fixed by glutaraldehyde perfusion fixation in vivo prior to sectioning. (d) shows a typical well developed subsarcolemmal bleb after 180 minutes of ischemia. The plasmalemma is fragmented and persists as circular profiles beneath the BL. A capillary lined by swollen endothelial cells (E) is in the field. Swollen mitochondria (M) of the underlying myocyte are also present. Mag A = 8750 X; B = 15000X; C = 62500X; and D = 19000X. (Reprinted with permission of the publishers of Reference [14]).

ischemia, only one-third of the pool remains. The degradation occurs [20] as a consequence of the action of adenylate kinase, which salvages the ~P of ADP and in the process forms AMP. The AMP is dephosphorylated to ADO by 5' nucleotidase. This ADO is deaminated by adenosine deaminase to INO. Once these nucleosides are formed, they diffuse from the myocyte and are essentially lost from the adenylate pool because little or no salvage synthesis can occur in the absence of ~P. When the myocardium is reperfused, interstitial INO and HPX are washed to the venous circulation where they can be detected in the coronary sinus.

Reversible and Irreversible Injury; Effect of Reperfusion: Reperfusion of the ischemic myocardium with arterial blood at any time up through 15 minutes of severe ischemia (Figure 4), results in salvage of the ischemic myocytes. By definition, these myocytes are *reversibly injured*. After 1-3 minutes, aerobic metabolism resumes; the adenylate charge is restored and the electrocardiographic changes of ischemia disappear. However, 4-5 days of reperfusion are required to restore the depleted adenine nucleotide pool to control levels [21]. Moreover, efficient contractile function does not recover for hours or days. This contractile defect is termed *stunning*.

Irreversibly injured myocardium exhibits the same metabolic changes seen in reversible injury but they are more marked (Figure 4). Lactate, H^+, INO, and HX levels are high and virtually no ~P remains in the tissue. AMP is the principal adenine nucleotide. Moreover, this tissue exhibits striking changes in ultrastructure (Figure 5). All mitochondria are swollen and contain amorphous matrix densities. In addition, the sarcolemma exhibits areas of disruption of the plasmalemma (Figure 5). The breaks in the cell membrane are considered to be the structural event that dictates that ischemic injury is irreversible [16].

Reperfusion of irreversibly injured tissue with arterial blood after 40 minutes of ischemia results in striking changes in the tissue. It swells enormously; total tissue water increases by 21% after only two minutes of arterial reflow. In addition, the mitochondria of the irreversibly injured myocytes accumulate calcium phosphate (CP) in the form of

hydroxyapatite; the Ca content of the tissue increases by a factor of 10 or more. Finally, the entry of extracellular Ca^{2+} into the tissue results in massive contraction of the myocytes. This is termed *contraction-band necrosis* and develops simultaneously with the edema and mitochondrial Ca uptake [22].

If irreversibly injured tissue is reperfused later in the irreversible phase, e.g., after 90 minutes of ischemia, areas of *no-reflow* are common in the middle of the zone of severe ischemia [23]. These are considered to be the result of ischemic damage to the microvasculature [23]. The endothelium becomes massively swollen and perfusion of the tissue often is impossible. In addition, myocyte swelling and the development of cardiac rigor impair reflow [24,25]. Thus, in addition to areas of no-reflow, some of the tissue exhibits *low-reflow*.

Another complication of microvascular damage is the appearance of areas of hemorrhage [10,26] in the zone of severe ischemia. The timing of the hemorrhage has not been established. It clearly is present after three or more hours of reperfusion have passed but it is not known how soon it develops after the onset of reperfusion. It seems likely that it develops in seconds or minutes but no direct data is available to support this hypothesis.

Transmural Gradient of Cell Death in Ischemia: Irreversible injury develops quickly in the zone of severe ischemia in the dog heart. Similar speedy development of necrosis appears in hearts with severe ischemia transmurally, i.e. hearts with no collateral flow. Here necrosis is complete throughout most of the myocardium at risk by the time 90 minutes of ischemia have passed [11]. Moreover, this tissue can be salvaged totally by reperfusion of arterial blood only within the first 15-20 minutes of the ischemia.

On the other hand, the transmural gradient of ischemia found in the average dog heart with collaterals, is associated with a characteristic transmural gradient of cell death such that the mid- and subepicardial myocardium survive for as long 3-6 hours. In some cases, cell death is not completed until 6 hours of ischemia have passed. In fact, myocytes in

areas of mild ischemia, such as those found in the subepicardial myocardium, may survive permanently. These concepts are summarized in Figure 6.

Analysis of the data in Figure 6 shows clearly that reperfusion at 40 minutes converts a potentially transmural infarct into a subendocardial infarct while reperfusion at three hours salvages some reversibly injured myocardium in the mid-and subepicardial region (Figure 6). However, little tissue usually is available to salvage after 3 hours of ischemia have passed. Note that there is no difference in the size of an acute myocardial infarct perfused after six hours of ischemia and that induced by 96 hours of permanent ischemia.

Thus, the major determinants of infarct size in the dog heart are, first, the amount of myocardium at risk, and second, the volume of collateral flow. Hemodynamic factors are important as well but contribute relatively little to the eventual size of an infarct. The most important variable determining how much of the area at risk undergoes infarction is the volume of collateral flow to the area at risk.

Role of Collateral Arterial Flow in Delaying Myocyte Death: The exact means by which collateral flow delays death of ischemic myocytes is unknown. It seems likely that it is due to improved energy production. Small quantities of 0_2 brought with the flow could allow some aerobic respiration with the result that much more ~P would be produced per unit of glucose metabolized to $C0_2$ and H_20 than is produced by anaerobic glycolysis. Also, the increased flow should reduce the osmotic load, perhaps wash out inhibitors of anaerobic glycolysis that have accumulated in the sarcoplasm, and moreover, provide exogenous glucose as substrate to relieve the demand placed on the limited supply of glycogen. In any event, the aphorism, "*a little collateral flow goes a long way toward maintaining viability,*" clearly is true.

Although collateral channels enlarge when stimulated by changes in pressure such as that brought about by acute coronary occlusion [11] in the dog heart or arterial stenosis secondary to coronary artery atherosclerosis in man, this change requires about four days to complete

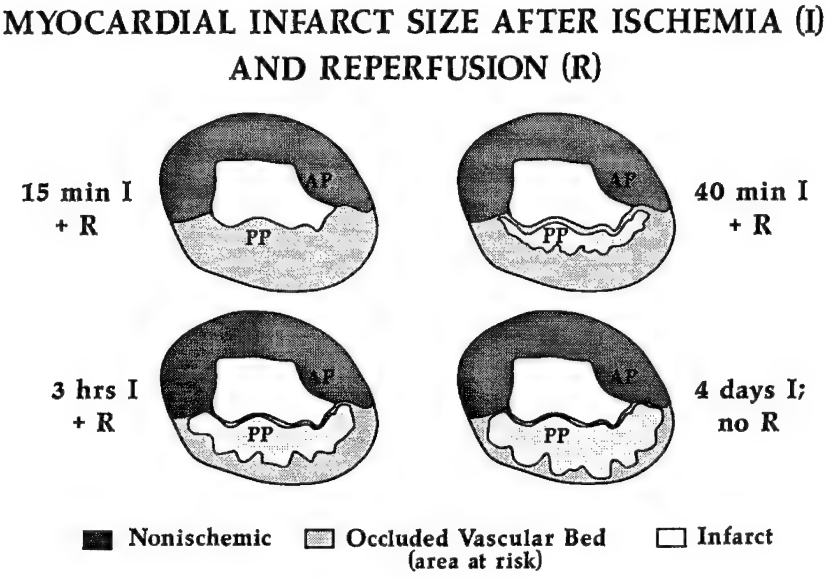

FIGURE 6. Progression of cell death vs time after circumflex coronary occlusion in dogs. The location of infarcts induced by different periods of ischemia followed by reperfusion is illustrated. Necrosis occurs first in the subendocardial myocardium. With longer occlusions, a wave front of cell death moves from the subendocardial zone across the wall to involve progressively more of the transmural thickness of the ischemic zone. In contrast, the lateral margins in the subendocardial region of the infarct are established as early as 40 minutes after occlusion and are clearly defined by the anatomic boundaries of the ischemic bed. Early reperfusion salvages some of the ischemic tissue and thereby limits the transmural extent of infarct. AP, anterior papillary muscle; PP, posterior papillary muscle (Modified from Ref. 26). (Reprinted with permission of the publishers of Reference [22]).

[27]. A theoretical means to increase effective collateral flow in the animal hearts containing significant collaterals would be to reduce blood viscosity while maintaining its oxygen carrying capacity. This might be achieved by replacing erythrocytes with an 0_2 carrying substrate such as hemoglobin or perfluorocarbon. Under this set of circumstances, ischemic myocytes should receive more collateral flow and thereby be less ischemic and survive for a longer period than they would have if no interventions had been attempted. There is some preliminary evidence available in the brain which suggests that this may be true [28]. So far, this hypothesis has not been tested thoroughly in the heart.

Testing Interventions on Myocardial Infarct Size: In order to test if a therapeutic agent has a beneficial effect on myocardial ischemic injury, one needs to know how big an infarct would have been if no intervention been employed. A standard protocol has been developed for this purpose; it is described in detail in Circulation Research [9] and is usually referred to as the AMPIM protocol (Animal Models for Protecting Ischemic Myocardium). In the dog heart, the effects of therapy can be assessed in groups of 8-12 treated and 8-12 control dogs by controlling for the chief determinants of myocardial infarct size, i.e., the amount of myocardium-at-risk and the volume of arterial collateral flow. The experiments should be done with the operator blinded as to the nature of the intervention. In addition, one should aim to hold hemodynamic factors as constant as possible between the treated and control groups.

The results of studies using the AMPIM technique have shown that it is possible to delay, but not to prevent myocyte death with a number of interventions. One of the most dramatic forms of protection is that brought about by preconditioning the myocardium with ischemia. The effects of this therapy are shown in Figure 7. Note the characteristic inverse relationship between collateral flow and infarct size as a % of the myocardium-at-risk in the control group. The lower the collateral flow, the larger the infarct and vice versa. Note, however, that at any level of collateral flow, infarcts induced by 40 minutes of ischemia in

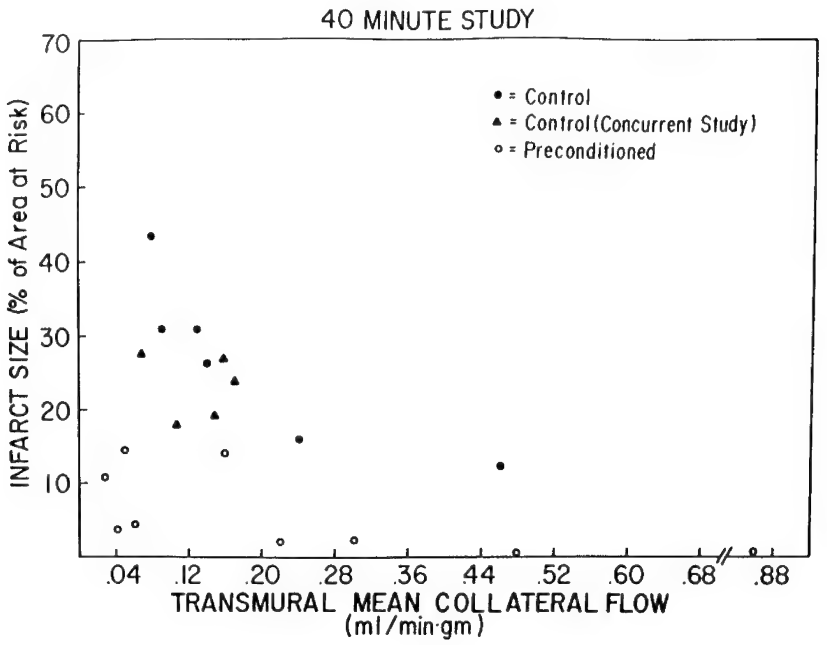

FIGURE 7. Infarct size vs collateral blood flow after 40 minutes of ischemia and four days of reperfusion. The relationship between infarct size, normalized as a percentage of the area at risk, and transmural mean collateral blood measured at 20 minutes into the 40 minute coronary occlusion is illustrated. In control dogs (closed circles and triangles), animals with greater collateral flow had smaller infarcts and vice versa. However, among dogs preconditioned with four 5 minute episodes of ischemia and reperfusion (open circles), all animals had small infarcts, irrespective of collateral blood flow. Each point represents one animal. The closed triangles represent control animals from another study, treated identically, which are included to better define the relationship between flow and necrosis in control hearts. (Reprinted with permission of the publishers of Reference [2]).

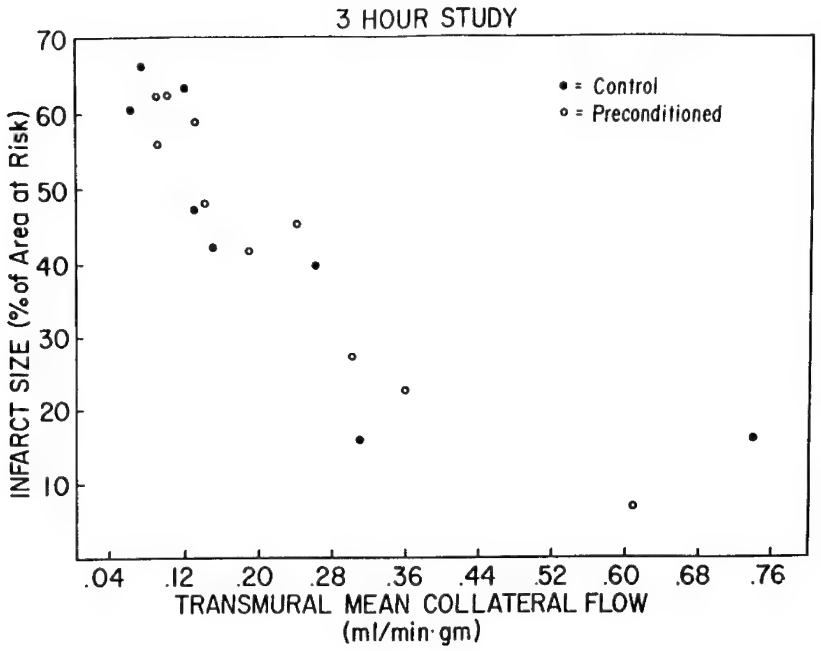

FIGURE 8. Infarct size vs collateral flow after 3 hours of ischemia and 4 days of reperfusion. The relationship between infarct size, as a percentage of the area at risk, and subepicardial collateral blood flow measured 105 minutes into the 3 hour occlusion is illustrated. For both control (closed circles) and preconditioned (open circles) animals, there is a close inverse relationship between infarct size and collateral flow. Also, there is no difference between the preconditioned and control groups with respect to this relationship. (Reprinted with permission of the publishers of Reference 2)

preconditioned myocardium are smaller than those observed in untreated heart.

The same inverse relationship between infarct size as a % of the myocardium-at-risk and collateral flow exists at three hours but the infarcts are much larger (Figure 8). On average, 3 hours of ischemia induces necrosis of about 70% of the myocardium-at risk. The clear circles

on this graph are from animals preconditioned with the same ischemia and reperfusion protocol used in Figure 8. Note that preconditioning failed to induce any beneficial effect. This means that preconditioning can delay but cannot prevent ischemic myocyte death.

An enormous number of studies have reported beneficial effects of a variety of therapies on myocardial infarct size in the dog heart. Results of these studies are questionable if they did not control for collateral flow and the volume of the myocardium-at-risk. Note that these factors would not need to be controlled if the intervention induced total protection and no necrosis developed in the treated group. However, up to the present time, no intervention has been successful in achieving this therapeutic effect.

Lethal Reperfusion Injury: Although reperfusion with arterial blood is the only means available to salvage ischemic myocardium, it is possible that some aspect of the process of reperfusion kills myocytes that were alive at the time of reperfusion. Such cell death is termed *lethal reperfusion injury* [29]. A likely scenario would be the development of new foci of ischemia in the reperfused myocardium due to occlusion of capillaries with leukocytes infiltrating the area or to leukocyte derived free radical mediated damage to the endothelium [14,30-32]. Although this scenario seems possible, evidence that it does or does not occur, has been contradictory. Perhaps this is because lethal reperfusion injury involves too few myocytes to be detected consistently. The lethal reperfusion injury concept remains an important unsolved problem [33]. It is important because, if it occurs, it seems likely that a therapy could be developed to prevent its development and thereby to salvage more myocytes by reperfusion.

There also is evidence that *sublethal reperfusion injury* exists in reversibly injured myocardium. For example, myocardium exposed to 15 minutes of ischemia is stunned. For periods of 24 or more hours or more, it does not contract as efficiently as control myocardium. Bolli [34] has shown that about 50% of this dysfunction is explained by damage due to oxygen derived free radicals acting on the ischemic myocytes. The source

of these free radicals is unknown. However, since fewer leukocytes are present in reversibly injured myocardium than were present prior to the onset of ischemia [35], it seems unlikely that the free radicals originate from leukocytes. Other forms of sublethal reperfusion injury may exist but have not yet been identified clearly.

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ART. CELLS, BLOOD SUBS., AND IMMOB. BIOTECH., 22(2), 279-284 (1994)

OVERVIEW OF THE HYPOXIA AND CANCER SESSION AT THE FIFTH INTERNATIONAL SYMPOSIUM ON BLOOD SUBSTITUTES

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The search for an ideal agent to improve response of cancer cells to radiation and chemotherapy without increasing normal tissue toxicity has resulted in the discovery of a number of pharmacologic and physical agents which modify the biologic effect of ionizing radiation and chemotherapeutic agents. Oxygen is perhaps the simplest and most powerful of these agents. Two papers presented at the general session on hypoxia and cancer suggested the ability of perfluorochemicals to improve radiation and chemotherapy response in cancer therapy.

In 1921 Holthusen reported that *ascaris* eggs were resistant to the effects of radiation if exposed under hypoxic conditions [1]. The lack of reproducible, quantitative radiation assays limited understanding of the powerful and central effect oxygen played in the fixation of radiation damage in biologic systems. H.L. Grey and his colleagues, utilizing an in vivo system, demonstrated that at all levels of survival or effect, three times as much radiation was necessary when the test system was hypoxic compared to well oxygenated conditions [2]. Howard-Flanders and Alper defined the level of necessary hypoxia and showed that the full oxygen effect was achieved with partial pressures of oxygen in the range of 15-20 torr [3].

Thomlinson and Grey suggested that within human tumors there might be regions of hypoxia with pO_2's less than 15 torr [4]. They produced histologic sections of tumors with large areas of necrosis and tumor cords about 100 μ wide. Ian Tannock showed that

the diffusion path length of oxygen from tumor capillaries was less than 100 μ and that the micrographs obtained by Thomlinson and Grey could be explained by a deficient tumor capillary vasculature and the presence of hypoxic, but viable tumor cells at the end of the oxygen path [5].

These observations were underscored when Powers and Tolmach demonstrated a biphasic radiation survival curve for a murine lymphosarcoma [6]. The low dose portion of the survival curve was dominated by the radiosensitive response of the majority of the tumor which was well-oxygenated. However, approximately 1% of the tumor cells were relatively radioresistant. These presumably hypoxic survivors determined the more shallow "tail" of the survival curve which had a slope approximately 1/3 that of the initial dose-response slope. Utilizing in vivo cell survival assays, the percentage of hypoxic cells within a number of experimental tumor systems have since been measured to be anywhere from 1 to 50% [7].

There has been much controversy within the radiation oncology community about the relevance of these experimental results to the clinical situation. At this conference, Dr. PAUL OKUNIEFF reviewed the data that suggested the presence of relevant hypoxic cells within human tumors. He reminded the audience of the indirect evidence of improved response to radiation in the hyperbaric oxygen trials of the British Medical Research Council [8]. In cervical and head and neck cancer, survival was increased when patients were irradiated in a 3 atm. O_2 environment. He also reviewed the trials of Bush and his colleagues from Toronto where transfusion of anemic patients to a hemoglobin level of greater than 12 g/100 cc resulted in improved local control of cervical cancer [9]. However, Okunieff suggested that direct and indirect measurement of tumor oxygenation (and its correlation with survival) was the most efficient way to assess the effect of hypoxia modifying cancer therapies. His paper concentrated on methods of direct and indirect estimation of tumor oxygenation. He reviewed electrode measurements, PET scanning, nitroimidazole binding, ^{19}F-NMR, and spectrophotometric analyses. All methods had advantages and disadvantages.

Most work to date has been done with electrode measurements. These polarographic methods tend to consume O_2 and thus be potentially inaccurate. In the past this method was inherently invasive, and it was therefore difficult to get repeated measurements from the same region to demonstrate the effect of any therapeutic

intervention. Okunieff reported on the impressive clinical material from Vaupel and his colleagues from Mainz, Germany [10]. Using the adanced Eppendorf Histograph with a microelectrode probe, Vaupel was able to show that 14% of the tumor measurements in patients with breast cancer were less than 5 torr. In addition, the patients with the low O_2 measurements were less likely to have their tumors sterilized with radiation than those patients with well-oxygenated tumors.

PET scanning oximetry utilizes a ^{18}F-nitroimidazole which is bound to hypoxic cells. This method is non-invasive and can estimate the hypoxic fraction of tumors. It results in excellent spatial resolution of hypoxic regions. This technique can obtain measurements from deep anatomic sites, unlike electrode and spectrographic techniques. However this indirect method may be sensitive to metabolic states other than hypoxia leading to false estimation of oxygen levels. Also, since the nitroimidazole binding is irreversible, repeated measurements, after a therapeutic intervention may be impossible.

Okunieff also reviewed the use of ^{31}P-MRS and ^{19}F-MRS to estimate oxygenation. The former method uses the ratio of the ATP phosphorus peak or the phosphodiester peak to the inorganic phosphorus peak. When the O_2 level is normal, the ATP/Pi ratio is high. In hypoxic zones, the ATP/Pi ratio is markedly decreased. By manipulating the environment to decrease hypoxia, the tumor can be used as its own control. The measurements can be done repeatedly and are available at sites deep to the physical probe. However spatial resolution is somewhat problematic at deep sites and there is quenching of measurements by normal tissue phosphorus compounds.

Okunieff described the work of Mason and his colleagues from Dallas utilizing the direct relationship between the ^{19}F-MRS relaxation time (R1) and oxygen tension. Mason has measured the oxygen tension within a rat tumor in vivo to be approximately 40 torr. This work will be presented elsewhere in these proceedings.

The other main lecture on neoplasia was given by Prof. EMIL FREI who discussed the state of cancer therapeutics. Frei advanced a framework in which the probability of cancer cure was directly related to 1) the presence of a number of active therapeutic agents, 2) the use of these agents in combination, 3) appropriate dose or scheduling of agents, and 4) advances in supportive care. The chance for cure was inversely related to: 1) tumor burden, 2) prior radiotherapy or chemotherapy, 3) lack of response to prior treatment, and 4) toxicity. He illustrated this framework by

summarizing the progress in the treatment of childhood acute lymphatic leukemia. Cure rates were sequentially increased when physicians and scientists recognized: 1) the need for increased drug dose and agents which had non-competitive toxicity, 2) the need for maintenance (adjuvant) therapy, 3) that drug scheduling influences normal tissue toxicity, and 4) that the blood-brain barrier was a physiologic sanctuary for tumor cells in the central nervous system, requiring changes in drug delivery strategies. With these sequential modifications, cure rates for childhood ALL increased from 20% in the early 1950's to over 70% today. Frei argues that that similar analyses of the reasons for failure of solid tumor therapy can result in significant improvement in therapeutic efficacy for the common solid malignancies.

Frei cataloged what he considered to be the most exciting advances in cancer pharmacology over the past few years. New drugs that merited special attention included: 1) natural agents such as taxol, 2) agents directed against the DNA repair enzymes topoisomerase I and II, 3) liposome formulations of older agents such as liposome encapsulated doxorubicin, and 4) anthrapyrazoles. Frei felt that an understanding of oncogene product action may lead to modifiers, or inhibitors of abnormal gene signal transduction leading to decreased abnormal proliferation. He described the production of monoclonal antibodies as a new class of agents specific to tumor cells: "magic bullets." Also discussed were antiproliferation and differentiation agents. A prototype of these was trans-retinoic acid which caused complete remissions in acute promyelocytic leukemias characterized by chromosome 15-17 translocations.

Finally, Frei discussed the problem of hypoxia in solid tumors and how its modification might lead to increased tumor cell kill by conventional chemotherapy. Frei cited the work of Jain [11] who showed that deficient tumor vasculature and lymphatics lead to tumor swelling with subsequent further decrease in tumor capillary inflow and secondary hypoxia. He also cited the work of Sartorelli and Teicher [12] who demonstrated that hypoxia was associated with alkylating agent resistance. This resistance allowed for increased tumor cell survival with attendant clonal evolution to heterogeneity and acquired drug resistance. Frei maintained that the hypoxia might be modifiable by the use of perfluorochemicals and oxygen breathing with improvement in alkylating agent activity and increased cell kill. Frei concluded that it was important to measure the increased tumor oxygentation associated with the administration of

perfluorochemicals. His group in Boston intended to use the Eppendorf oxygen electrode and histograph to investigate the ability of a perfluorooctyl bromide emulsion (Oxygent™ [13, 14]) to act as an oxygen carrier for this purpose.

The papers of OKUNIEFF and FREI anticipated the presentations by S. ROCKWELL and B. TEICHER on fluorocarbons in cancer therapy. These investigators described the use of perfluorooctyl bromide emulsions to improve radiation and chemotherapy action. Posters by M.GUICHARD and G. SCHWARTZ discussed the use of the Eppendorf electrode in murine and clinical situations. R. MASON contrasted the effect of the a perfluorotripropylamine emulsion with a perfluorooctyl bromide emulsion on the ^{19}F-NMR relaxation time (R1). These papers are part of these proceedings.

The enhancement of murine tumor response to chemotherapy and radiotherapy with the first generation perfluorochemical emulsion Fluosol-DA® was not confirmed in the human trials with Fluosol, possibly due to the inability to deliver sufficient concentrations of the emulsion to the tumor vasculature. The strategies discussed in this symposium increase the likelihood of success with second-generation preparations. Human tumor and xenograft measurements, such as those described by Guichard, Mason, Okunieff, Rockwell, and Teicher will identify those patients whose tumors are likely to require enhancement of oxygen delivery. Phase I pharmacology studies, such as those described by Schwartz will lead to an understanding of the maximally tolerated fractionated dose of these compounds. Thus, by targeting human tumors that have significant hypoxia with adequate doses of a fluorocarbon oxygen carrier, the importance of tumor hypoxia as a cause of therapy resistance will be determined.

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ART. CELLS, BLOOD SUBS., AND IMMOB. BIOTECH., 22(2), 285-294 (1994)

TECHNICAL IMPROVEMENTS IN THE STAGING OF CANCER: THE ROLE OF IMAGING AND CONTRAST AGENTS

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ABSTRACT: Despite advances in cancer diagnosis, there has been little impact upon outcome. This may be a consequence of the exceptional heterogeneity in cancers, especially their cell types, perfusion, oxygenation, and metabolic circumstances. Better therapeutic plans could require better characterization of individual tumors in individual patients. For some tumors, tissue characterization by sophisticated histologic analysis of biopsy samples may improve staging. However, noninvasive staging is more acceptable to patients and may be more comfortably repeated in the course of monitoring therapeutic regimens. Several imaging applications may serve these goals. First, new contrast agents may allow accurate cancer detection in regional lymph nodes. High resolution CT with perfluorocarbon lymphography is in the clinical trial stage. This

could presage minimally invasive ablation of cancer in lymph nodes, as well. Second, new agents will better define local and metastatic cancers, also impacting standard and minimally invasive treatments. Finally, imaging methods may be able to measure perfusion and metabolism in small volumes *in vivo*, as well as estimate important local pharmacokinetics of therapeutics.

ANATOMIC IMAGING

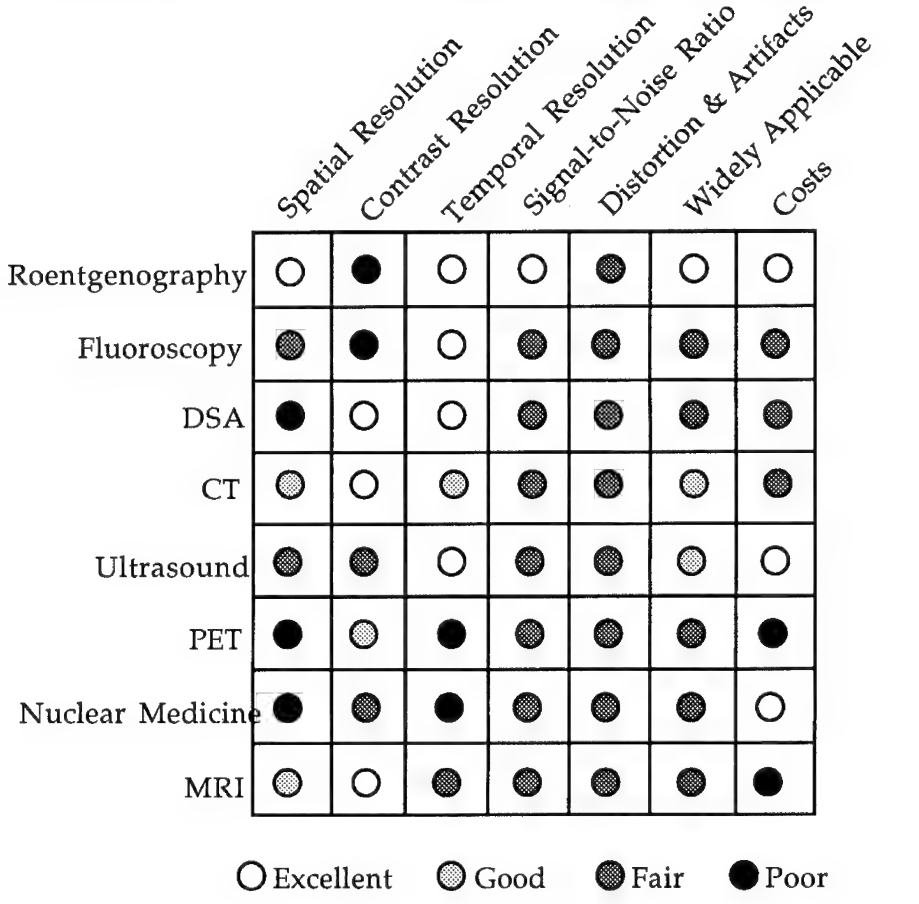
Since the description of x-rays nearly 100 years ago, imaging specialists have been asked to locate cancer within patients. Clinical classification of cancer began with the League of Nations Health Organization in 1929 and continues to be refined and improved. Over the same interval, there continue to be rapid changes in the performance, availability, and cost of imaging technologies with the addition of ultrasound, computed tomography, radioactive isotope imaging, and magnetic resonance imaging (TABLE I.)

The focus for staging cancer with imaging modalities is localization of neoplasia utilizing the TNM system (FIGURE 1). In general, we believe that cancer arises from a local process within one or a few cells, grows slowly *in situ*, initially spreads via local lymphatics, and sooner or later gains access to the systemic circulation. Successful metastasis requires favorable cancer cell characteristics and implantation sites. Thus, dissemination is slow and usually sparse. Both temporal evolution and focal metastatic growth favor the use of imaging for staging of cancer.

Much progress has been made in conceiving methods to identify tumor markers in the blood or identifying patients with

TABLE I.

Imaging Characteristics of Selected Diagnostic Systems



significantly increased risk of developing cancer. These strategies have resulted in the discovery of barely macroscopic or even nonpalpable malignancies - tumors at their earliest stage. A necessary concommitant of this early recognition of neoplasia is the development of imaging strategies to guide the localization of the very small volume of malignant cells.

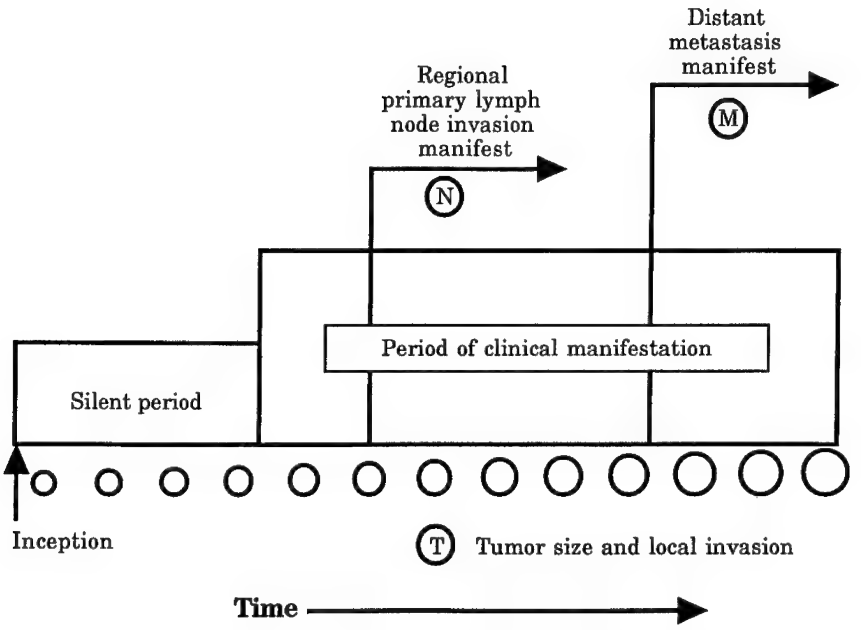


FIGURE 1: General Developmental Events for Cancer. (Modified from American Joint Committee on Cancer).

Fortunately, modern diagnostic imaging is usually able to identify primary tumors before they attain the size that metastasis becomes likely. Imaging often guides biopsy and even ablation of these very small, primary tumors.

When cell type and location are known, imaging is then used to assess local lymph nodes and expected sites of metastatic spread. Lymph nodes are usually small (< 1 cm), numerous (500-800 total in man), and homogeneous to high resolution tomographic imaging (CT or MRI). Although cancer growth in nodes displaces normal node histology and can increase node size, the determination of node size is a poor staging criteria.

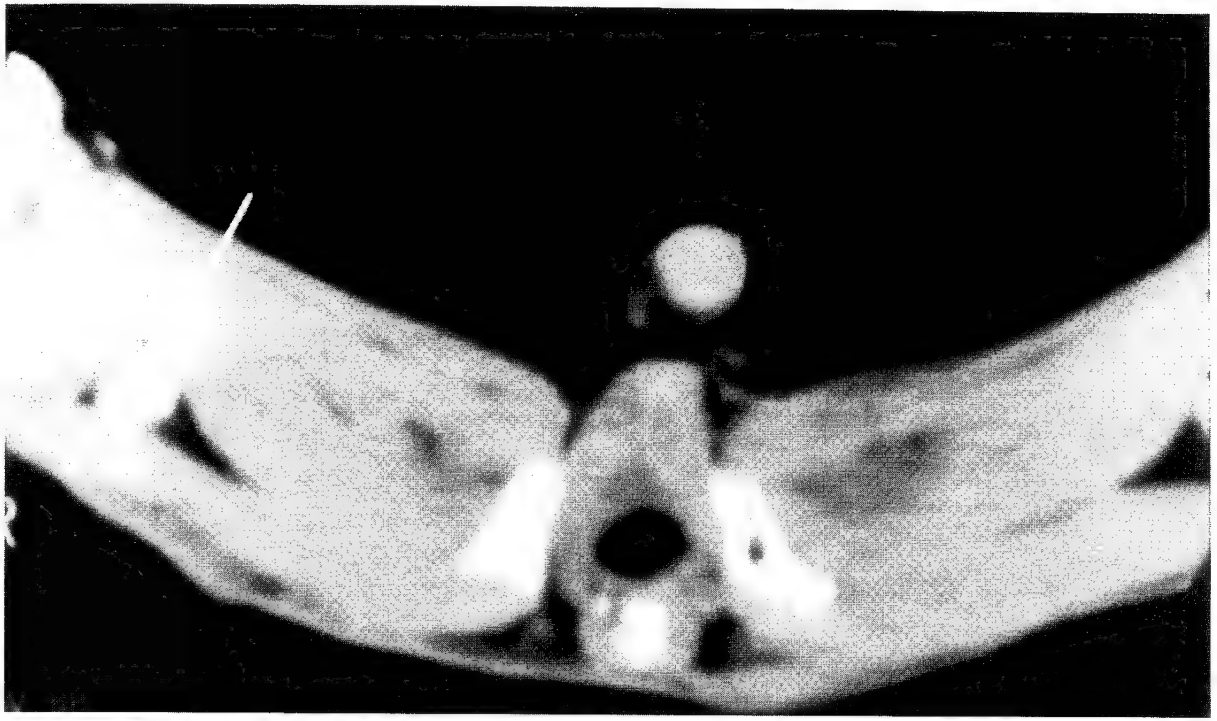


FIGURE 2: Percutaneous Lymphography of Popliteal Node. In this normal rabbit, 0.5 ml of PFOB emulsion (Imagent^{LN}, Alliance Pharmaceutical Corp.) was injected subcutaneously in the right forepaw. A CT scan taken 9 days later shows normal intranodal architecture (arrow).

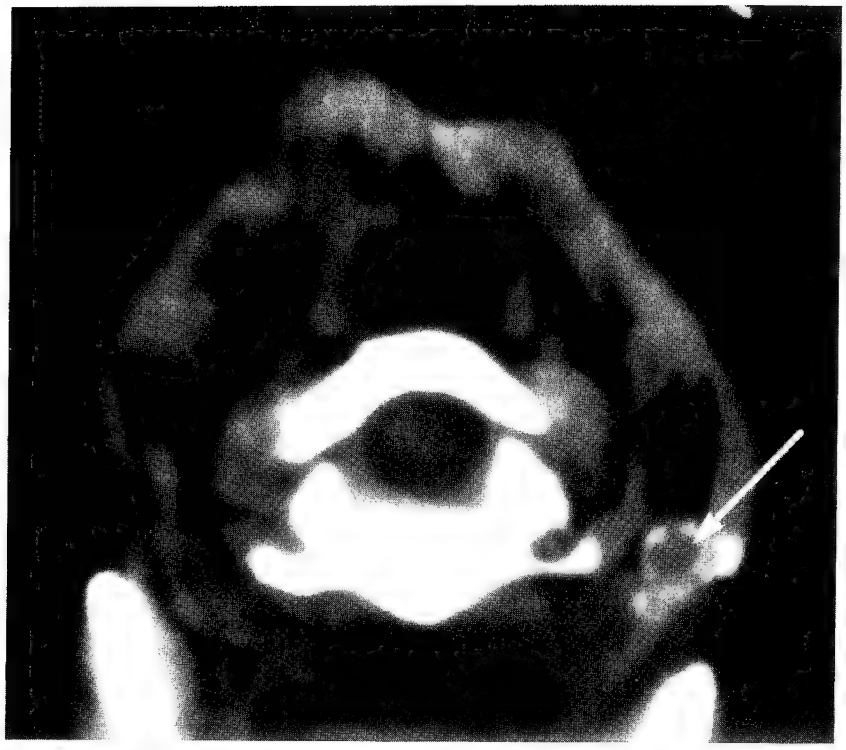


FIGURE 3: Cancer in Lymph Node. Percutaneous lymphography shows the filling defects due to cancerous replacement of lymph node (arrow). Courtesy of J. Bruneton.

Lymphangiography can visualize normal and abnormal lymph node architecture, but uses unsafe contrast agents, surgical instillation, and visualizes only a limited number of nodes. Recently, our laboratory has been able to identify intranodal architecture with volumetric CT and percutaneous administration of radioopaque emulsions [1] or nanoparticulates [2]. These agents are in early clinical trials and could be an

important improvement in the staging of cancer (FIGURES 2 & 3).

Contrast agents for CT and MRI are also providing more accurate identification of parenchymal metastases, especially in brain and liver, with major impact upon the selection of cancer treatment.

PHYSIOLOGIC IMAGING

Although imaging of the distribution of cancer is central to staging, I believe that dynamic imaging has the potential to characterize the physiology of cancer in ways that tailor therapeutics. We look to the insights of tumor angiogenesis [3] and tumor perfusion [4-6] to plan our investigation.

Both CT and MRI have become able to perform fast imaging with high spatial resolution. Each also has safe contrast agents that can be administered as an intravenous bolus. Applying the principles of indicator dilution to small regions of interest, it becomes possible to measure important tumor parameters *in vivo* [7-9].

Angiogenesis And Dynamic Imaging

The pioneering work of Judah Folkman has now provided a sound basis for understanding when tumors have generated the local vascularity to sustain growth and allow metastasis. In an elegant series of studies, they have found that the metastatic potential of breast cancer is closely related to abnormal capillary count and density of the margin of the primary tumor [3]. In this junctional zone, vascular density and permeability sustain tumor growth.

TABLE II.

Heterogeneity in Cancer

- Cell type(s)
- Perfusion
- Oxygenation
- Metabolism
- Therapeutic response

Although imaging may assist in identifying the breast lesion for biopsy and histologic staging using Folkman's indices, dynamic breast imaging with MRI and its special indicator, GdDTPA, may provide the same information. The high capillary density of malignant tumors would account for an early, rapid washing of MR indicator while benign tumors with less angiogenesis would have delayed and slower contrast enhancement [10]. If validated, then this staging by dynamic imaging also offers characterization of the entire tumor without biopsy.

Tumor Biology and Dynamic Imaging

The laboratory of Rakesh Jain has studied the perfusion characteristics of tumors and his efforts explain why cancers are so difficult to treat (TABLE II). Along with the angiogenesis comes adverse hydrodynamics for drug delivery. At first glance, the enhanced vascular permeability of tumors is cause for optimism, but there is no hydrostatic or oncotic pressures to sustain drug delivery. Further, diffusion distances can be huge for reasonable blood drug half life and macromolecules are

especially penalized [11]. Finally, tumor physiology is markedly heterogeneous between tumors, and even within the same tumor varies incredibly in space and time [5,6].

Dynamic imaging has all the attributes necessary to characterize local physiology. Spatial resolution with MRI and CT is now less than 0.1 ml, temporal resolution resolves the expected range of tumor perfusion in ml/min/gm, the entire tumor volume can be characterized, an appropriate range of indicators (size, composition) is achievable, and the tumor characterization is repeatable as often as necessary. The process is also quite acceptable to patients. At a minimum, dynamic imaging of tumors will be an unusually powerful research tool and work has begun [10,12].

SUMMARY

Imaging technology has reached the stage where it must be considered as new treatments for cancer are developed. This potential is no less important for drug therapy than it is for ablations by standard surgical or minimally invasive interventions.

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THE POTENTIAL ROLE OF PERFLUOROCHEMICALS (PFCS) IN DIAGNOSTIC IMAGING

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ABSTRACT

Although perfluorochemicals (PFCs) are known for their ability to carry oxygen, they are the most versatile and only universal contrast agents with important applications using x-ray, ultrasound, or magnetic resonance (MR). The characteristics that make them unique diagnostic agents are lack of hydrogen atoms, immiscibility with water, low surface tension, compressibility, and long intravascular persistence when emulsified and given IV. When made radiopaque, they are visible with x-ray computed tomography (CT) and standard radiography. Because the neat liquid is inert it can be ingested, instilled in the lung, or introduced into any hollow organ to image the lumen without untoward effects. The long intravascular persistence allows the imaging of blood vessels and vascularized tissues. Small or deep vessels become visible on Color Doppler Imaging and angiographic images of any vascular tree including the coronaries can be rendered from the serial CT images. As PFCs accumulate within RE cells, specific liver and spleen enhancement is achieved allowing the detection of small tumors within these organs. When injected interstitially, the particles find their way to the draining lymphnodes providing detail of the internal architecture to detect the presence or absence of tumor involvement on both CT and sonography. Using ^{19}F MR, tissue perfusion and tissue pO_2 measurements can be achieved. As can be seen, the applications of PFC in diagnosis are vast, unique, and important. These new capabilities will carry radiological tools to new horizons.

TABLE I

Applications of Neat Radiopaque PFCs

Magnetic Resonance	Radiography	Computed Tomography
• Oral Contrast	• Gastrointestinal imaging	• High resolution imaging of lung parenchyma
• Sat Pad™	• Urinary bladder	• Oral Contrast
• Rectal imaging	• Sinography	
• Urinary bladder imaging		

INTRODUCTION

Perfluorochemicals (PFCs) are a class of compounds composed essentially of carbon and fluorine atoms. PFCs, like oil, are immiscible with water and cannot be given intravenously (IV) unless emulsified. They are inert, and have high gas solubility, low surface tension, and very low toxicity when ingested or inhaled [1,2]. They actually behave like a liquefied gas. Some are extremely volatile, like freons, whereas others are solid. They accumulate in human tissues when inhaled, ingested, or given intravenously and are eliminated by expiration. The length of time they remain in the body is related to their molecular weight and vapor pressure, the more volatile they are the shorter their half-life, which can range from days to years [3,4]. Fluosol® (Alpha Therapeutic Corp., Los Angeles, CA) and Imagent® BP (Alliance Pharmaceutical Corp. San Diego, CA) are two emulsions that have been given IV to human subjects [5,6] without serious side effects. In addition to the well published diagnostic applications of PFC emulsions for all 3 imaging modalities CT, ultrasound, and magnetic resonance (MR), there are many diagnostic applications for neat PFC as well. This report will outline the potential applications known at the time of this writing for neat as well as emulsions of PFCs when used to diagnose disease.

As neat liquids, PFCs possess unique physical and chemical properties that make them widely applicable in diagnostic applications (Table I). They are immiscible with water and possess low surface tension. These characteristics make them practical to use since they can traverse the bowel more rapidly than water

based materials when ingested [7]. Further, because PFCs do not become diluted and do not concentrate as they propagate through the bowel lumen, their effect is not influenced by bowel content, hydration state, or bowel disease. Thus, radiopaque PFCs such as perflubron (USAN for perfluorooctyl bromide) may serve a role in gastroenterography particularly when bowel obstruction is suspected [7]. Clinical trials are underway to evaluate the utility of Imagent® GI in the setting of bowel obstruction (Alliance Pharmaceutical Corp.).

Applications of neat PFCs in conjunction with CT are being developed their potential utility in this setting is still unclear. One potential application described by Wolfson et al in these proceedings is the high resolution imaging of lung parenchyma following the instillation of neat perflubron in the trachea [8]. They were able to visualize bronchioles not previously resolved. This capability may prove to be useful in the assessment of distal airway disease as in bronchiolitis obliterans.

Because PFCs contain no hydrogen atoms, they emit no signal at the proton resonance frequency appearing black on MR imaging (Figure 1) [9, 10]. When ingested, Imagent GI served as a bowel markers when the abdominal or pelvic regions were imaged with MR. More importantly, it was found that perflubron possesses magnetic susceptibility similar to that of tissues [11]. The absence of susceptibility differences at perflubron/tissue interfaces allowed a detailed view of mucosal surfaces [12]. The same principle applies when Imagent GI is instilled in the rectum where in addition to the added mucosal detail, it is able to distend the rectum which is typically collapsed, improving the anatomic delineation of lesions. In a similar application, replacing the bright signal of urine in the bladder with the black signal of PFCs on T2-weighted sequences improved the dynamic range of the scanner and provided greater internal architectural detail of bladder tumors [Unpublished data].

The lack of magnetic susceptibility difference with tissues stimulated the development of Sat Pad™ (Alliance Pharmaceutical Corp.) which consists of PFC filled pads used to improve image quality on MRI. Sat Pad converts the irregular anatomic contour of the body to a cylinder improving magnetic homogeneity and therefore image quality particularly when fat saturation techniques are employed.

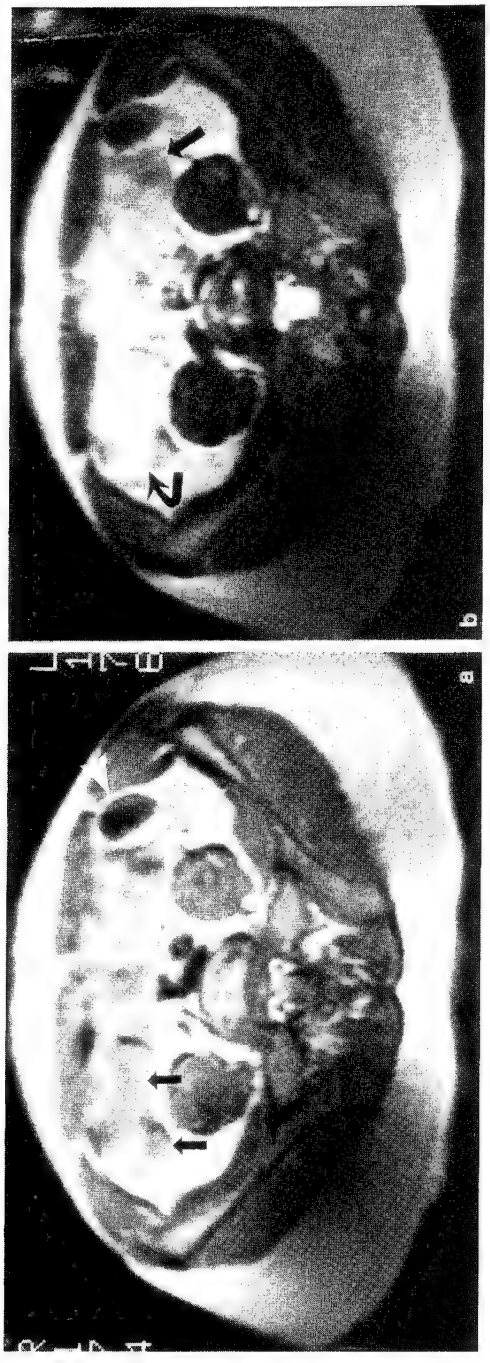

b
a

FIGURE 1: Axial scans were obtained over the mid-abdomen of a volunteer using TR=2 sec and TE=20 (a) or TE=70 (b) prior to (a and b) or following Imagent GI (c and d) using the same TR and TE. Note that where air is present before contrast bowel is dark (arrow head in (a)). Remainder of bowel is intermediate in signal pre-contrast (arrows in a and b). Water containing bowel is bright on T2-weighted images (curved arrow in b). After ingestion, Imagent GI fills all bowel loops which now appear dark in signal (arrows in c and d). (Published with permission of Mattrey [ ] and *AJR*).

TABLE II

Applications of Emulsions of PFCs

Magnetic Resonance	Sonography	Computed Tomography
• ^{19}F imaging	• Vascular imaging	• CT Angiography
• pO_2 measurements	• Assess tissue perfusion	• Tissue perfusion imaging
	• Liver/Spleen imaging	• Liver/Spleen imaging
	• Tumor imaging	• Tumor imaging
	• Indirect lymphography	• Indirect lymphography
	• Assess renal function	• Image renal osmotic gradient

Further, it replaces the air/skin interface with an air/PFC interface which is further removed from the skin surface. Because air has a large magnetic susceptibility difference with tissue, the displacement of the air/tissue interface away from the area of interest increases detail on the MR image. For a more in-depth description of this application and for illustrations please refer to the paper by Eilenberg et al in these proceedings [13].

Magnetic Resonance Imaging

Fluorine is the next best nucleus for MR applications after hydrogen, since it has 100% natural isotopic abundance and has an 83% sensitivity relative to hydrogen. Although all PFCs can be detected, they must possess certain characteristics to be practical. Some of their properties must include their ability to produce stable injectable emulsions and the magnetic spectrum should ideally consist of a single ^{19}F peak or the resonances should be sufficiently shifted from each other to allow their separation when using MR imaging equipment. Perflubron has 3 peaks, 2 single peaks (CF_2 and CF_3) that are shifted 60 and 40ppm from a quintuplet. Either or both single peaks are typically used since they can be easily isolated and both possess sufficiently long T2 relaxation times to be practical. MR imaging or spectroscopy of ^{19}F in PFCs can be used to estimate tissue pO_2 non-invasively. PFCs can carry a significant amount of O_2, which owing to its unpaired electrons, is paramagnetic. Therefore the presence of O_2 in PFC shortens both T1 and T2 such that 1/T1 and 1/T2 change linearly with O_2 content

[14]. Tissue pO_2 can be estimated by appropriate MR techniques, since pO_2 in PFCs is passively carried and is in equilibrium with tissue pO_2 [15], and the ^{19}F relaxation rate is linearly related to pO_2 dissolved in the PFC [14]. Although it is possible to quantitate pO_2 in any tissue in the body, blood pO_2 estimates are technically difficult because flow also affects signal which is used to calculate T1 and T2 relaxation times. However, PFC particles accumulate in liver, spleen, bone marrow, and tumor macrophages to become fixed within these tissues providing the unique capability to monitor intracellular pO_2. For more in depth discussion on this topic and illustrations please refer to the two manuscripts in these proceedings by Mason [16] and Guo et al [17].

X-ray Computed Tomography (CT)

While water soluble iodine-based urographic contrast agents are ideal for renal CT scanning, they are suboptimal for use to image the blood pool and various organs on CT. These agents are lost to the extravascular space because they quickly diffuse into and equilibrate with the interstitial fluid. The amount lost to the interstitium on the first pass through the capillaries has been estimated to be 20% under high flow conditions [18] and this loss increases further as flow decreases or becomes sluggish as is observed in tumors [19]. Since perflubron emulsion particles remain intravascular, the dose can be titrated to provide the blood enhancement desired on CT and the degree of enhancement will be the same throughout the arteries, veins, and cardiac chambers [20]. With the several-hour blood half-life of perflubron particles, this enhancement persists long enough to allow ample time for CT imaging (Figure 2). Tissues enhance to a degree commensurate with their blood volume allowing the quantitation of their blood content [21]. Blood pool enhancement of tissues with perflubron emulsion on CT, aside from its higher spatial resolution, is comparable to labeled-red blood cell blood pool scanning in nuclear medicine. It is therefore expected that perflubron emulsion on CT should allow the differentiation of intrahepatic tumors from hemangiomas, since the former have less blood than liver and the latter are essentially a vascular pool [22].

EOE-13 (Ethiodized Oil Emulsion), like perflubron emulsion,, is taken up by the RE cells of the liver and spleen [23, 24] but unlike perflubron does not

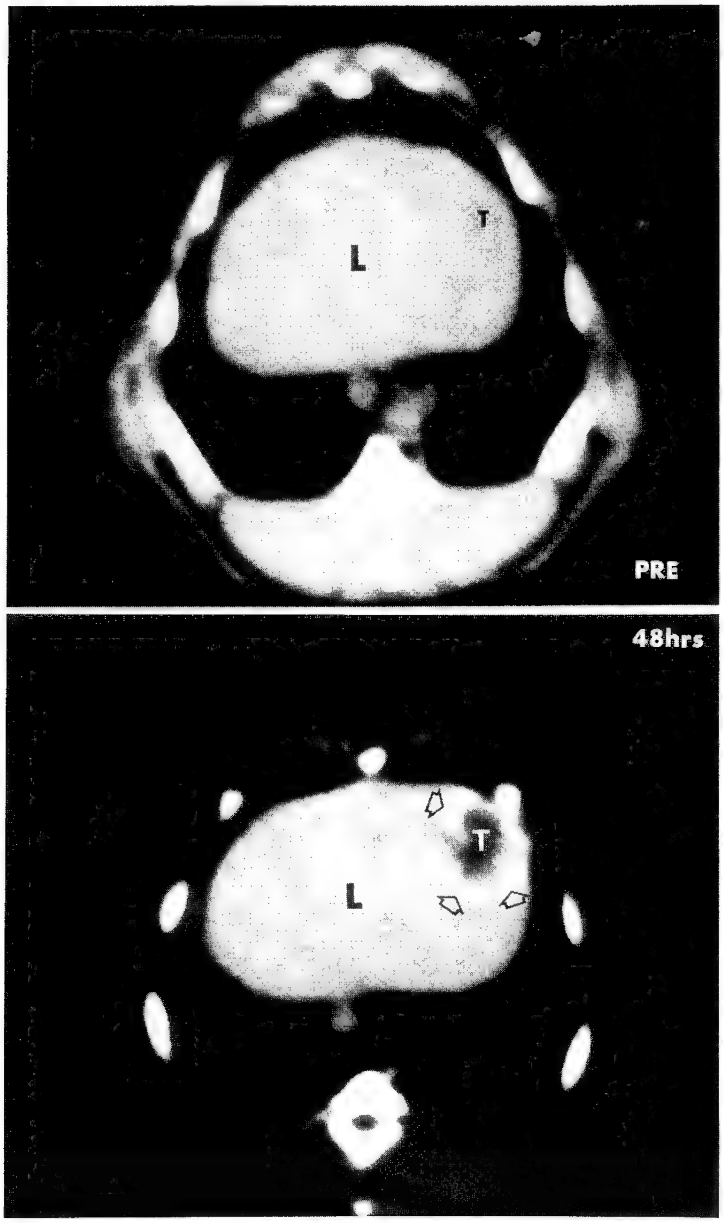
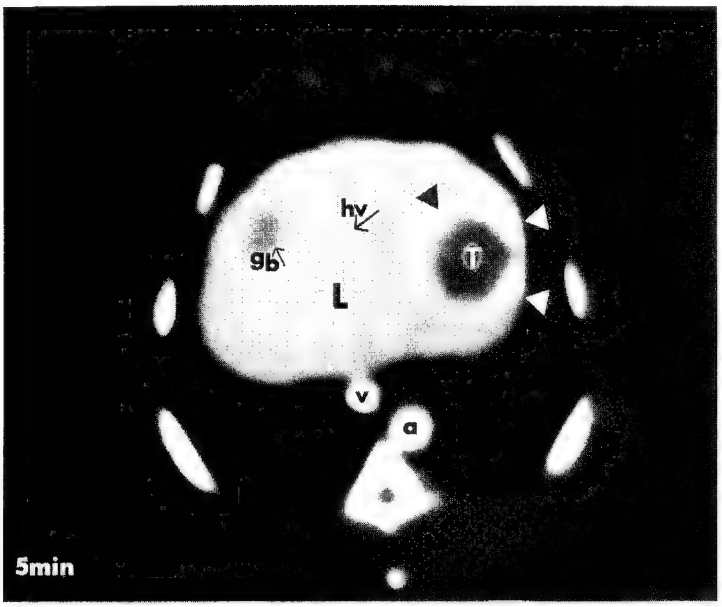


FIGURE 2: Transverse CT scans of a rabbit with VX2 tumor (T) implanted in the liver (L) that could not be seen pre-contrast. 5 min after infusion on 5 ml/kg of perflubron emulsion, there is liver (L), aorta (a), vena cava (v), and hepatic vein (hv) enhancement. The gall bladder (gb) and intrahepatic tumor (T) became visible as they fail to enhance relative to the liver. 48 hours later, tumor rim enhancement (open arrows) can be observed at the rim of the tumor (T). The lower right hand image is the anatomic slice taken at approximately the same level.



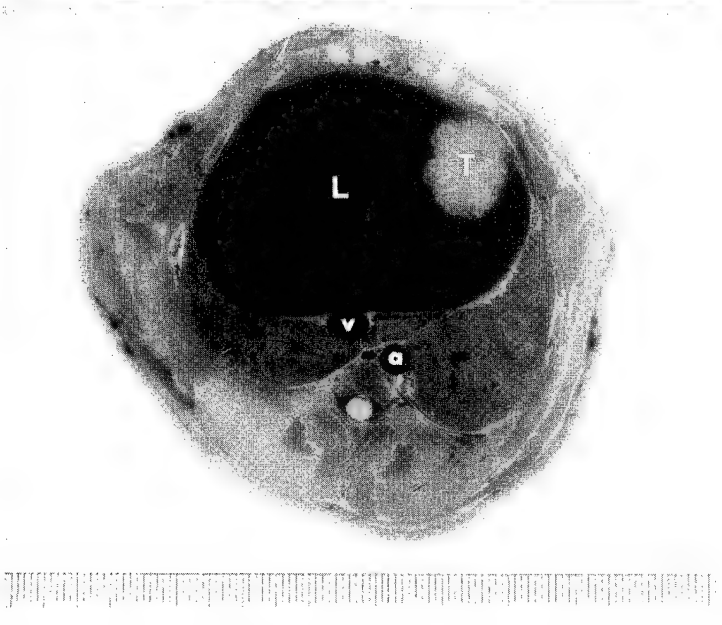


FIGURE 2 Continued

enhance blood vessels. It was shown to have a sensitivity of 90% which is considered to be the best of all CT techniques in detecting liver lesions [25]. The reason for the less than perfect sensitivity is because small lesions were confused with comparable sized intrahepatic vessels and vice versa [26]. The simultaneous enhancement of the vascular space with perflubron renders lesions the only unopacified structures within the liver, potentially providing greater than the 90% liver metastasis detection rate that was achieved with EOE-13. Further, the diffuse enhancement of both liver and intrahepatic vessels provide an opportunity for image segmentation in three-dimensional analysis of the liver to automate the calculation of tumor and liver volume. Automating tumor burden calculation should help in the management and follow up of patients with primary or metastatic liver disease. For further detail and illustrations please refer to the paper by Steinbach et al in these proceedings [27].

It appears that 1.5ml/kg of Imagent BP (a 90% w/v emulsion of perflubron) may suffice to produce blood and liver enhancement for tumor detection as described by Baker et al in these proceedings [28]. In their study, significant liver and blood enhancement occurred allowing the detection of small tumors. However, unlike the higher doses, tumor rim enhancement was not observed. Whether tumor enhancement will be detected at this dose in man is not yet clear. A clinical trial aimed at assessing the efficacy of 1.5ml/kg of Imagent BP in human subjects is presently underway. The development of more radiopaque PFCs such as those with an iodine in-lieu of a bromine atom is underway as described by Sanchez et al in these proceedings [29]. Although it has been speculated that iodine containing PFCs are unstable and toxic, Sanchez et al showed that the LD_{50} to diagnostic dose ratio in rats doubled when compared to a perflubron emulsion of similar formulation as Imagent BP.

Within minutes to hours after the infusion of perflubron emulsion, enhancement of abscess wall and tumors begins and peaks at 1-4 days [30]. Accumulation of perflubron particles in these sites is thought to occur by either transcapillary leak through abnormal neoplastic or inflammatory vascular beds, or by accumulation of perflubron-filled macrophages, or both. That transcapillary leakage occurs is evidenced by tumor rim enhancement minutes after infusion [31]

and the presence of perflubron particles in the perivascular space when lecithin is stained with Oil-Red-O [32]. By 48 hours, all of the perflubron in these sites is within macrophages that are then present in large numbers when compared to control [23,33]. It is not clear how these perflubron-filled macrophages accumulate in lesions. They may have taken up the perflubron particles elsewhere, became activated as has been suggested [34], and accumulated in immunologically active sites, or, they may have been resident or recruited to these sites to phagocytose the particles present in the interstitium. That the former mechanism may be true is evidenced by the fact that the enhancement of the abscess wall increased between days 4 and 10 after infusion at a time when the blood had trace amounts of perflubron [35].

Lesion enhancement has been documented by both CT and sonography after the administration of perflubron emulsion (Figure 2) [23, 31, 33, 36-38]. In fact, it appears that perflubron particles accumulate in any region where macrophages are found including tumors [23, 31, 36, 37, 39], abscesses [30, 33, 35], and injured [40] or infarcted [38] tissues. This accumulation leads to enhancement of the area on CT in proportion to the degree of inflammation [40]. An application of great clinical potential may be the use of perflubron emulsions as CT contrast agents to improve the detection of abscesses. In rabbits in which hepatic and peritoneal abscesses were induced, IV infusion of perflubron emulsion produced dense enhancement of the abscess wall on CT 2 days after infusion [33]. While liquefied centers of hepatic abscesses could be seen prior to infusion, the enhancement produced by perflubron emulsion made it possible to determine the extent of inflammation [33, 35]. Peritoneal abscesses on the other hand could not be distinguished from bowel prior to the infusion of perflubron emulsion but were detected in all rabbits after infusion owing to the intense rim enhancement produced [33]. It was also shown that the infusion of a large dose of perflubron emulsion (5g/kg) did not affect the body's response to infection in that abscess size and mortality were similar to saline control [35].

When perflubron emulsion particles (Imagent® LN, a 60% perflubron emulsion) are injected subcutaneously or intramuscularly directly in the interstitium, they are removed by two mechanisms: direct entry of the particles into

lymphatic vessels and phagocytoses by macrophages recruited to the site. In these proceedings is a study by Ikomi et al which demonstrates that the method of elimination is by direct flux of free particles in the first few days following the injection of Imagent LN and by macrophages later [41]. The removal of particles by either method results in their accumulation in the local lymph nodes draining the region markedly enhancing their interior. This concept was first described by Wolf et al using a rabbit model [42]. That enhancement of normal nodes with Imagent LN occurs in man was shown by Hanna et al in these proceedings [43]. Using a rabbit model with metastatic VX2 tumor to retroperitoneal nodes, we showed that when 0.5ml of Imagent LN was injected in the foot pad and intramuscularly in the thigh, retroperitoneal lymph node enhancement was detected. The pattern and degree of enhancement allowed the distinction of normal, hyperplastic, and neoplastic nodes. Normal nodes were small and enhanced markedly. Reactive nodes were enlarged and enhanced homogeneously and significantly but to a lesser degree than normal nodes. Neoplastic nodes were enlarged but enhanced faintly and hetrogeneously [Unpublished data]. This capability should allow the assessment of lymph node chains not accessible to direct lymphography such as axillary and cervical nodes as is observed with breast and head and neck malignancies.

Sonography

PFCs are effective contrast agents for sonography [36, 37]. Their echogenicity is due to their high density (1.9 g/ml) and low acoustic velocity (600 meters / sec.), imparting an acoustic impedance difference of 30% with tissues. Since impedance difference determines the brightness of the echo which determines echogenicity, and since the difference in impedance among tissues (except for fat) ranges from 1 to 5%, PFCs are highly reflective.

Doppler signals and their color rendition enhance significantly as a result of perflubron emulsion infusion [44]. This effect lasts for hours due to the long blood half-life of perflubron particles. The signal to noise ratio increases allowing Doppler signals, including color, to become detectable from submillimeter vessels as well as vessels not seen on the gray scale image [44, 45]. This capability should have a significant impact on deep Doppler applications where small or deep vessels reflect weak signals.

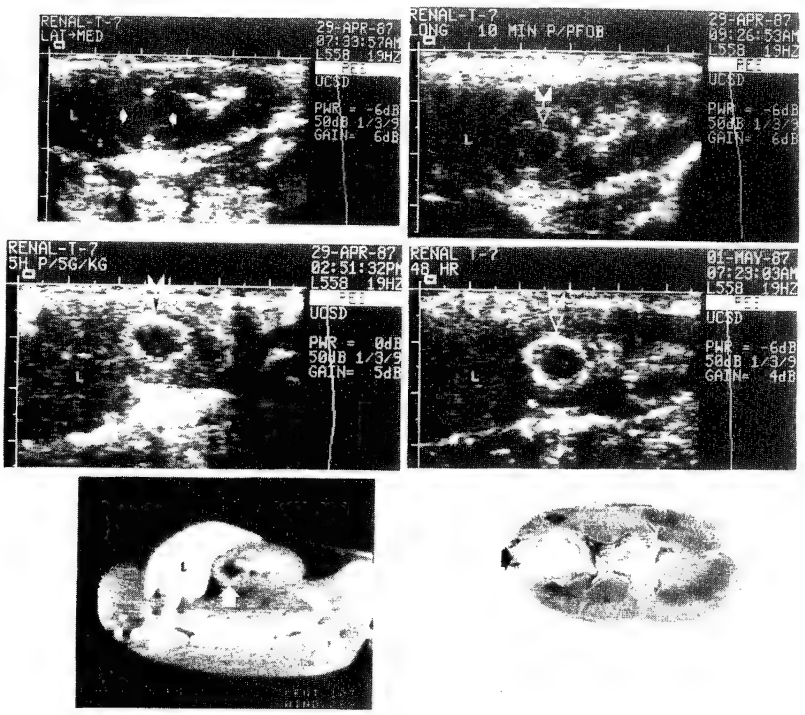


FIGURE 3: Longitudinal sonograms taken over the right kidney where a VX2 tumor was implanted in the upper pole (lower left). Before contrast, the tumor (arrows) was faintly seen. Within 10 min following infusion (upper right), the tumor become hypoechoic relative to the remainder of the kidney which enhanced following perflubron emulsion infusion. Also note the faint rim seen about the tumor. At 5 hours (mid left) and 48 hours later (mid right), the rim enhancement becomes more intense. Note the excellent correlation between the rim enhancement seen on CT and sonography. The anatomic detail observed in the ultrasound images (upper four), correlate with the CT images and the photograph of the cut specimen.

Perflubron emulsions also enhance tissue echogenicity during its vascular phase immediately following infusion (Figure 3) [31, 45]. The degree of enhancement is commensurate with degree of blood volume contained in the tissue. Hypovascular renal tumors that have the same or greater echogenicity than the kidney, become less echogenic immediately after perflubron emulsion infusion [31]. This is also true of liver tumors and renal infarction [45]. Increased echogenicity in proportion to the degree of vascularity may allow sonography to estimate tissue perfusion, visualize areas of infarction and tumors. It was observed on CT that the renal medulla, although less vascular than cortex, enhances to a much greater degree than the cortex when it is known that PFCs are neither excreted or accumulated by the kidney. This phenomenon was later shown to be due to the osmotic gradient which concentrated the particles within the vasa recta of the renal pyramid providing a visual depiction of the concentrating ability of the kidney [46]. Because perflubron emulsion enhances the echogenicity of perfused tissues, medullary enhancement was also observed on sonography. This phenomenon allowed sonography for the first time to image the concentrating ability of the kidney and to recognize when the osmotic gradient was lost as was observed with furosemide [47], acute tubular necrosis [47], and ureteral obstruction [48].

Once perflubron particles become stationary in tissues, either because of leak from abnormal capillaries or because of phagocytosis by macrophages, they markedly enhance the region where they have accumulated (Figure 3). This can be observed in the liver, spleen or tumors [36-39, 49]. In humans, both Imagent BP and Fluosol produced significant tumor enhancement allowing the detection of previously missed lesions as well as liver and spleen enhancement 24 hours after a dose of 1.5g/kg or greater allowing the visualization of unenhanced tumors [39]. Although Fluosol and perflubron emulsion produce nearly identical tissue enhancement once made stationary, their effect during the capillary phase is different [50]. Fluosol failed to increase Doppler signal, visualize flow on gray-scale, and enhance perfused tissues when compared to perflubron emulsion despite the fact the perflubron emulsion was diluted from 100 to 20% to match the PFC concentration in Fluosol [50]. It appears that this phenomenon may be formulation dependent [50].

When perflubron emulsion is administered interstitially and the particles accumulate in local lymph nodes, regions of accumulation become echogenic highlighting filling defects produced by tumors. In a study where Imagent LN was injected in the foot pad and in the thigh muscle of rabbits with metastatic VX2 tumor to retroperitoneal nodes, sonography was able to distinguish reactive from neoplastic nodes [Unpublished data].

CONCLUSION

PFCs have a wide variety of diagnostic applications in MR, CT, sonography, and plain radiography making them the most versatile and only universal contrast agents. The capabilities added to each of these imaging techniques are unique and clinically important, advancing each modality to a new horizon and raising the diagnostic capabilities to new heights.

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PERFLUOROCHEMICAL LIQUID AS A RESPIRATORY MEDIUM

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ABSTRACT

The use of perfluorochemical (PFC) liquids to facilitate or support respiration has been under study for several decades. The low surface tension and high respiratory gas solubility of liquid PFC enable adequate oxygenation and carbon dioxide removal at low insufflation pressures relative to gas ventilation in the immature or injured lung. Because liquid ventilation homogeneously inflates the lung and improves V/Q matching it has been studied as a vehicle for delivering biologically active agents to the lung tissues and systemic circulation. More recently, we have shown the utility of highly opaque PFC liquids as a high resolution computed tomographic (HRCT) bronchographic contrast agent either during LV or gas breathing after tracheal instillation of small quantities of PFC.

As a result of extensive experimental work in premature animals as well as lung injury models, liquid PFC ventilation has been recently implemented as an investigational therapy for severe respiratory distress in human infants. This manuscript summarizes the physiological principles and applications of LV as well as the results of initial investigational clinical studies in human neonates with severe respiratory distress.

INTRODUCTION

Numerous investigators have shown that perfluorochemicals (PFC), as either pure inert liquids [1-7] or emulsions [8-11] of these liquids, can

dissolve adequate oxygen and carbon dioxide to support respiration of entire mammals, isolated organs, and tissues. All of the evidence to date indicates that the respiratory system plays a key role in many of the biomedical applications of PFC liquids. With respect to emulsions, the lungs provide the primary route of PFC elimination from the body, whereas in liquid ventilation (LV) the lungs are the main route of both entry and exit.

The biomedical application of liquid ventilation has been explored in animal models for over 3 decades [12]. More recently, clinical investigational trials [13,14] have shown that it is possible to maintain gas exchange in very immature and critically ill neonates using an abbreviated form of this modality- combined with gas ventilation (GV). This manuscript will discuss the physiology and methodology of LV techniques. The rationale and current status of animal and human experience will be explored. Finally, the broad based potential applications will be discussed, as well as the feasibility of LV techniques in the treatment of respiratory disease.

MATERIALS AND METHODS

Breathing Liquids

Several fluids have been explored as breathing media to support ventilation; liquid replaces nitrogen gas as the carrier for oxygen and carbon dioxide [15-17]. Winternitz and Smith [18] first used saline solution to treat victims of poisonous gas inhalation. Subsequently, saline was employed by investigators to explore the relationship between respiratory function and structure [19-21]. Work with this media produced a significant body of knowledge about lung tissue and surface tension properties, and led to our current understanding of pulmonary surfactant physiology.

Perfluorochemical liquids were first used by Clark to support normobaric respiration in 1966 [1]. Initially, a fluorocarbon product known as FX-80, manufactured by the 3M Company, was the most widely used fluids. Others such as Caroxin-D, Rimar 101, APF-140 and perflubron (perfluorooctyl bromide, PFOB) were also found suitable.

Ventilation Techniques

Liquid breathing investigations first employed either total body immersion or gravity- assisted ventilation from a reservoir to an intubated animal; these methods, however, proved inadequate for extended ventilation of experimental animals [1,12,22]. Subsequent trials confirmed that when mechanical ventilation was performed properly, oxygenation and ventilation could be effectively maintained in the liquid-filled lung for extended periods up to 30 hrs. (personal experience). There is no known

physiological limitation to this form of ventilation. This more recent data reflected improved liquids and ventilation methods [2,4,6,7,23]. In this regard, several groups have reported the combination of conventional ventilator therapy with perfluorocarbon-filling of the lungs in newborn piglets [24], neonatal lambs, rabbits and pigs [25-28], rabbits and cats with respiratory distress [29-31], and in premature infants and adults with respiratory failure [13,14,32]; this combined ventilation scheme was well tolerated and in some cases demonstrated improvement in pulmonary function.

RESULTS OF BIOMEDICAL APPLICATIONS

Surfactant Deficiency

The capability of LV to evenly and maximally reduce interfacial surface tension indicates that this treatment could make a potent contribution to the care of neonates with premature lung disease. Extensive premature animal data and initial studies in human infants point to the potential and feasibility of LV in this scenario [6,27,33-37]. The potential advantages of LV in the treatment of respiratory distress syndrome, however, extend beyond the surface tension-lowering characteristics. Filling the lungs with liquid can recruit atelectatic regions and distend the lung parenchyma, thereby improving diffusion surface area, ventilation and ventilation/perfusion matching [5,15].

One possible application of LV in premature lung disease would utilize this approach to "condition" the lung [38]. In this scenario, liquid would be used to recruit lung units, improve regional gas exchange, and possibly carry exogenous surfactant uniformly to all regions of the lung. Following this brief liquid ventilatory treatment, the patient would then be "recovered" to gas ventilation .

Pulmonary administration of drugs(PAD)

With lung disease and intrapulmonary shunting, systemically administered drugs are ineffectually delivered to the diseased portion of the lung. The delivery of agents to the pulmonary can be facilitated by administering biological agents through the alveolar surface of the lung during LV [39].

Recent studies have demonstrated differences in pulmonary and systemic responses elicited by pulmonary administered drugs (PAD) as compared to those elicited by intravenous administration [16,40]. Both PAD and IV administration of biological agents demonstrated dose-dependent responses to increasing levels including cholinergic response to acetylcholine, sympathomimetic responses to epinephrine, and alpha-adrenergic blockade response to priscoline (figure 1). These data showed

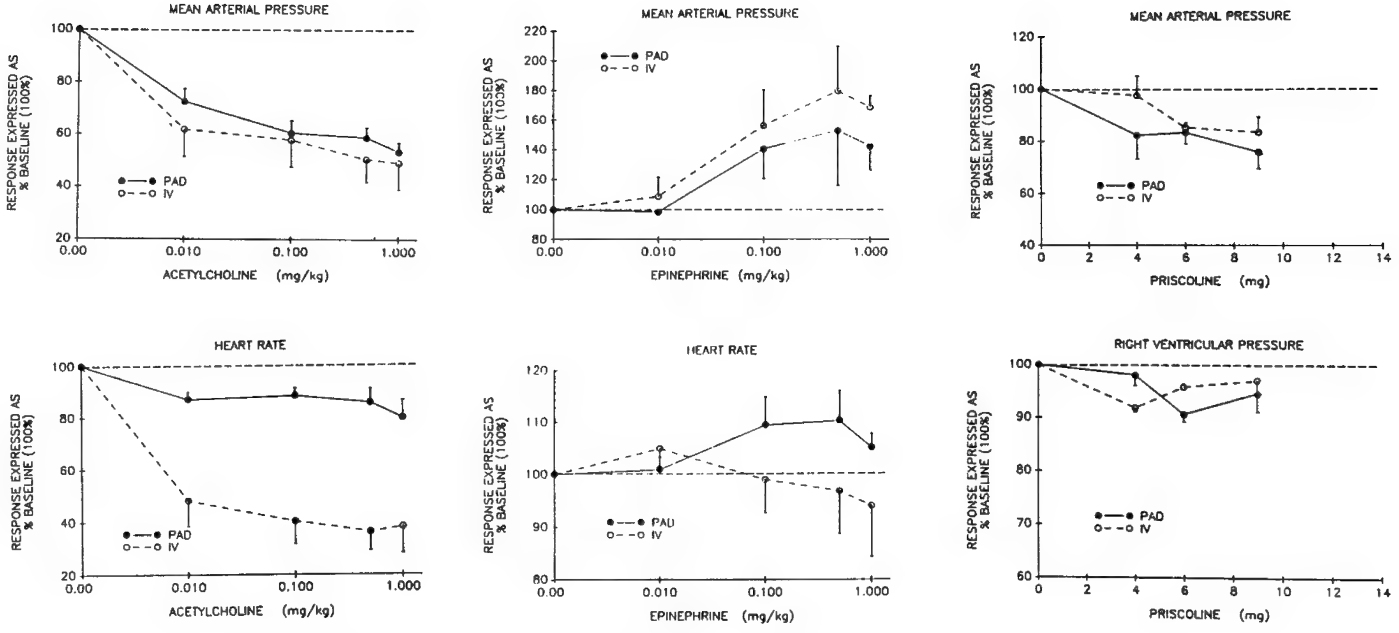

FIGURE 1: Physiologic responses to pulmonary administration of vasoactive drugs (PAD: filled circles) compared to responses elicited by intravenous administration (IV:open circles).[16]

that it is possible to deliver biologically active agents directly to the pulmonary system via perfluorochemical LV. Since these agents are insoluble in PFC, it is speculated that the agents are delivered through a convective mass transport mechanism. This technique may be useful to deliver both active and inactive agents for treating and/or diagnosing
pulmonary as well as systemic disorders such as respiratory distress syndrome (ie. exogenous surfactants, antibiotics, steroids, antioxidants), impaired pulmonary circulation (ie. diuretics, vasoactive agents for pulmonary hypertension) and lung cancer (ie. chemotherapy).

Lavage

Perfluorochemical liquid has been used to lavage preterm lambs [28], near-term meconium-stained lambs [36], and in cats with acute lung injury [30,31]. With respect to injured lungs, poor gas exchange, acidosis, and poor pulmonary compliance were present at birth and during GV of the meconium-stained lambs. Improvements were noted during LV in PaO_2, $PaCO_2$, alveolar-arterial oxygen gradient, and pulmonary compliance; and pulmonary blood flow was more uniform.

In more recent studies, it was shown that cats with severe acute lung injury demonstrated improved gas exchange and pulmonary compliance for approximately 60 minutes after lung lavage with oxygenated PFC. These beneficial effects could be repeated during subsequent serial PFC lavages, in the face of a lung injury which caused 100% mortality in 4 hrs in the untreated control group [30,31].

Imaging

Perfluorochemical liquids, in particular perflubron, make excellent contrast media when instilled into anatomical structures such as the lungs [41-42, also see Wolfson et. al. in these proceedings]. In addition, their 19f nucleus configuration make these liquids uniquely suited for high-contrast imaging techniques used to monitor organ function, biochemical mechanisms, and therapeutic measures. Furthermore, fluorine corresponds to a proton image, thereby providing a way of assessing ventilation-perfusion functions in relationship to anatomical structure. The clinical impact of such capabilities is enhanced if one considers the potential for quantifying regional oxygen tensions by observing calibrated spin-lattice relaxation times. In this setting, fluorocarbons can be introduced using lung lavage, aerosol spray, or direct tracheal injection.

The acoustic attenuation in PFC liquids is substantially lower than that of tissue, making these liquids suitable for highlighting lung structure in ultrasound technology. Perfluorochemicals have the lowest sound

TABLE I: Clinical Profile of Infants

PATIENT	DIAGNOSIS	PREVIOUS ADDITIONAL Rx	AGE AT LV	INITIAL pH	RESPONSE TO LV
1	28 wks/RDS emphysema	High Frequency Ventilation	16 Days	7.21	Improved ABG Cl/Res for 4 Hrs
2	24 wks/RDS	Exogenous Surfactant	48 Hours	6.77	Transient increase in Cl
3	23 wks/RDS emphysema	Exogenous Surfactant	7 Hours	7.22	Increased Cl/pO2 for 2 hours
4	37 wks/MAS PPHN	High Frequency Ventilation Exogenous Surfactant	24 Hours	7.64	Increased Cl/pO2 for 5 Hours
5	34 wks pneumonia	High Frequency Ventilation Exogenous Surfactant	48 Hours	7.32	Transient increase in Cl/pO2
6	23 wks/RDS	High Frequency Ventilation Exogenous Surfactant	24 Hours	6.74	Transient Improvement in Cl/pO2/pCO2

speeds of any biomedical liquid; this factor in combination with their acoustic attenuation, afford a depth penetration capability ideal for ultrasound imaging.

Cancer

Another potential clinical application of the PFC filled lung is in the area of lung cancer treatment. Adjunctive hyperthermia is being used more and more to augment radiotherapy and chemotherapy. Sekins et al [43] have used PFC liquids to induce localized lung hyperthermia. This procedure is more efficient than alternative "whole body" heating in the following ways: 1) liquid as a propagation medium for ultrasound hyperthermia; 2) liquid ventilation to heat the treated lung section by convection; 3) liquid as a vehicle for chemotherapy (to concentrate dosage in the damaged areas; and 4) liquid hyperoxygenation for improving radiation results.

Investigational Clinical Studies With LV

The protocol for clinical LV was approved by the institutional review boards at Temple University Hospital and St. Christopher's Hospital for Children where the initial studies were conducted [13,14]. Neonates were

considered candidates for LV if they were not candidates for other conventional therapies or if these treatments had failed.

Six critically ill infants met criteria for LV as shown in Table 1. All infants had marked cardiovascular instability with periods of bradycardia and hypotension requiring resuscitative measures before LV. In addition, these infants had poor oxygenation and /or ventilation on high pulmonary inflation pressures, despite trials with several other therapies. Infants 4 and 5 had relatively normal $PaCO_2$ but were hypoxic due to their specific disease state. None of the infants had a recent pneumothorax.

Oxygenated (F_IO_2 = 1.0) liquid PFC (Rimar 101, Miteni, SPA Milano, Italy,) was placed in a calibrated inspiratory reservoir (R_I) which was suspended above the supine infant and was attached via a Y-piece to the endotracheal tube. Gas ventilation was interrupted to instill a liquid functional residual capacity (20-30 ml/kg) from the R_I; gas ventilation was then resumed. Liquid ventilation was then initiated (two, 3 min trials separated by 15 min of GV). Tidal volumes (15 ml/kg) of PFC were delivered to the lung via gravity assistance; and, similarly was removed from the lung through the remaining port of the y-connector into a calibrated expiratory reservoir which was suspended below the infant. Each tidal volume of liquid was held within the infant's lungs for 15 seconds at a frequency of 2-3/min.

Heart rate, blood pressure, hemoglobin-oxygen saturation , arterial blood gas tensions , and pulmonary function were evaluated during GV before and after LV.

All infants tolerated the LV procedure without difficulty. Although all infants had fluctuations in these vital signs(heart rate, hemoglobin saturation, and mean arterial blood pressure) ; the greatest observed fluctuations typically occurred during subsequent GV.

The percent change in pulmonary gas exchange and function after LV (while on GV) from baseline values for the six infants is shown in Figure 2. As shown, the response in oxygenation and respiratory compliance after LV was consistent; in spite of the severe degree of lung damage. After several hours, the condition of each infant began to deteriorate and no further improvements were observed with standard neonatal management. After several hours, all infants died of their underlying respiratory disease except for patient 4, whom survived to 3 months of age.

A chest x-ray taken immediately following LV (infant 1) revealed homogenous distribution of PFC liquid (seen as uniform radio-opacities) and no evidence of pneumomediastinum or pneumothorax. Inspection of the lungs at autopsy revealed severe hyaline membrane disease and

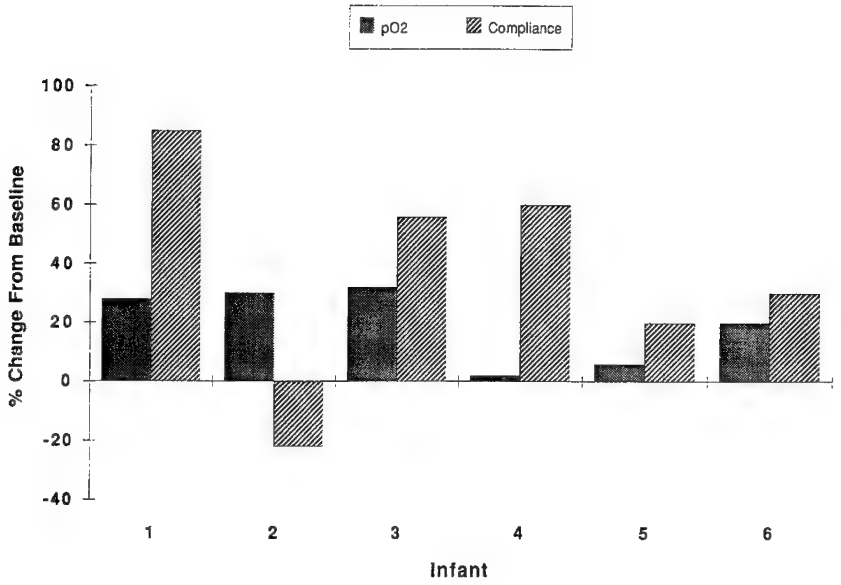

FIGURE 2: Percentage change from baseline in arterial blood oxygenation and pulmonary compliance after second cycle of liquid ventilation. [▮-PO_2; ▨- Compliance]

barotrauma; there was no evidence of PFC liquid in the pleural cavity. Histological analyses of lung sections obtained from liquid ventilated infants demonstrated a relatively larger proportion of clear, uniform, well-expanded, and thin-walled gas exchange spaces as compared to tissues obtained from age-matched infants who treated with conventional gas ventilation. Similarly, sections of liver, spleen, kidney and thymus were unremarkable and comparable to those obtained from gas ventilated infants [44].

Perfluorochemical uptake, biodistribution, and elimination was assessed in several of these infants by analyzing blood, tissue, and expired gas samples with electron capture gas chromatography [45]. The data demonstrated that PFC uptake and elimination was organ dependent and PFC levels in the blood were relatively saturated by 15 min. of LV. Also it was found that PFC concentration in the expired gas samples decayed exponentially to within control range by 8 hrs following LV. Other methods

of elimination such as transpiration through the skin and mechanisms which influence biodistribution and elimination of the perfluorochemical liquid including organ perfusion, lipid composition, perfluorochemical vapor pressure and ventilation/perfusion matching in the lung warrant further investigation.

DISCUSSION

Animal studies to date have demonstrated that insufflation of preterm lungs and acutely injured adult lungs with an oxygenated PFC liquid eliminates elevated interfacial surface tension. Therefore, in contrast to conventional GV, gas exchange and acid-base balance during LV can be more effectively achieved at lower and safer alveolar inflation pressures. In addition, the application of liquid ventilation techniques such as pulmonary administration of drugs, lavage procedures, and pulmonary imaging enhancement offers unique clinical advantages for the treatment at and diagnosis of several types of lung disease.

Preliminary findings from the clinical studies are encouraging in that all infants tolerated LV and demonstrated some residual improvement in pulmonary function after return to gas ventilation. Although all six infants ultimately died of their underlying respiratory disease, this trial does demonstrate that PFC ventilation can support gas exchange, even in preterm infants with severe and prolonged lung disease. For ventilation with perfluorochemicals to assume a role in clinical medicine, further studies are warranted.

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ISSUES AND PROBLEMS IN ORGAN PRESERVATION IN THE U.S.

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HISTORY OF MODERN TRANSPLANTATION

Organ transplantation is a highly visible therapeutic modality. Rarely has a medical discipline generated and sustained such public attention. The concept of transplantation, in its various forms, has been discussed throughout history. The modern era of transplantation began with the experiments of Hunter when he transplanted a cock's spur into its comb.(1) At the turn of the century, Carrel used the concept of transplantation to develop his innovative surgical techniques for suturing blood vessels,(2) which eventually lead to the transplantation of a whole dog head. Although the rejection process was not conceptualized by these studies, Carrel was the first to recognize that autografts survived longer than allografts. Carrel later collaborated with Lindberg to develop the first apparatus for organ preservation. The early attempts to perform kidney transplants, both experimentally and clinically, were aided by the work of Medawar, which demonstrated the role of the immune response. Using a skin graft model, Medawar developed the concept of initial and second-set

rejection.(3) Studies using a skin graft model demonstrated permanent survival between monozygotic twins and formed the basis of the first human transplant between monozygotic twins by Murray in 1954.(4)

In the 1940's Kolff designed the first dialysis machine using cellulose tubing.(5) The availability of heparin provided the opportunity to dialyze patients and their continued survival provided the population to support the developing renal transplantation technology. Chronic dialysis was developed by Scriber in 1958, and provided the opportunity to dialyze patients for longer periods of time.5 The growing population of patients with end-stage renal disease increased the demand for transplantation. In 1960, Hitchings and Elion of Burroughs Wellcome Research Laboratories produced 6-mercaptopurine (azathioprine).(6) The synergy between azathioprine and steroids lead to a dramatic increase in graft survival to approximately 50% at one year.

Experimental transplantation also experienced dramatic growth during this period. The common antigenicity of tissues was described by Dempster and Simonsen using a skin and kidney graft model; where either tissue could provide the sensitizing milieu for the other.(7) Other milestones included the first canine cardiac transplant by Shumway(8) and the first canine liver transplant(9) by Starzl. Extrarenal organ transplantation continued to develop and the years immediately prior the 1970's saw the first human heart and liver transplantations. Although rejection was still a major problem, the transplantation field grew rapidly through the 1970's. Kidney transplants became routine, liver and heart transplants were more widely performed and one year graft

survival was dramatically raised by as much as 20%. These advances in transplantation were largely made possible by the introduction in the 1970's of the immunosuppressive Cyclosporine A, a fungal peptide.(10)

The 1980's brought major increases in the number and variety of transplants. Clinical transplantation now encompasses kidney, pancreas, heart, heart/lung and liver grafts. The progress in transplantation during the 1980's has expanded the demand for organs to an unprecedented level.

DEMAND FOR ALLOGRAFTS

Transplantation is the therapy of choice for end-stage organ failure. It is lifesaving in the case of heart and liver transplants, and for renal patients enhances their quality of life, and is more cost effective. In 1987, 127,000 people were on dialysis and covered by the end-stage renal disease program of medicare. The number of people being added to the hemodialysis roles doubles approximately every 10 years. Only a fraction of this patient population is awaiting a transplant. There are approximately 30,000 patients presently on the National Transplant list.(11) This increase is in the face of an organ donor pool which has remained constant for 3 of the last 4 years, with a slight decrease in 1992.(11)

The major limiting factor in transplantation today is the shortage of transplantable organs. This persistent shortage is being addressed by the United Network for Organ Sharing (UNOS). UNOS has been awarded the government contract to operate the Organ Procurement and Transplantation Network (OPTN) and a scientific registry

for organ transplantation. All transplant programs and organ procurement organizations (OPO) must be approved by UNOS. The cost of transplantation in the United States is reimbursed by Medicare.

Most OPOs (75%) are hospital based while the rest are independent organizations. OPOs are a young service industry, 54% of which have been incorporated since 1986. Even with the significant increase in OPO activity, the donor rate per million population base has not significantly increased nor has it come close to the theoretical possibility of 20-30 donors per million population. The effect is a continuing increase in the demand for organs while the supply remains constant. Living-related organ donation for kidney grafts has steadily increased in the face of the severe organ shortage.

The organ shortage problem is now so severe that the UNOS has formed an ad hoc committee to develop an action plan to increase organ donation. This committee has developed seven white papers; which identify two new possible mechanisms for increasing the number of donors.(11) Presumed consent laws would assume that a person has agreed to be an organ donor unless the family explicitly states that the patient will not be a donor. Financial compensation has been suggested in the form of assistance for funeral costs, a flat fee or a tax credit. These two ideas are bold and are presently being debated.

THE ORGAN DONOR SHORTAGE

There are three major problem areas in organ donation/procurement:

1) limited organ donor pool
2) obtaining consent
3) preservation and associated problems

Most organs in the United States are procured from heartbeating cadavers. Heartbeating cadavers are patients who succumb to head trauma and are maintained on life-support systems. Heartbeating cadavers represent a small fraction of trauma patients, and therefore, represents a limited organ donor pool. The number of potential cadaver donors available for transplantation in the U.S. has been estimated in numerous studies, the most optimistic estimates are 55 donors/million/year or theoretically 11,200 donors giving 22,400 kidneys. The recent overall national organ retrieval rate, however, is only 16.5% of this figure.(11) Why only 16.5%? A study by the NY regional transplant program provided an informative explanation. In 1985 only 140 kidneys were procured in the NY metropolitan area (population of over 8×10^6). In contrast, 954 corneas were donated during the same year. Despite the adoption of the required request law, where next-of-kin must be approached for consent to donate in cases of brain death, the rate of kidney donation was only slightly increased to 172, while cornea donations increased to 1,702 donations.

These factors highlight the problems confronting family members in deciding organ donations. When families are not confronted with the decision of terminating life-support systems, these families are much more likely to make the decision to donate, as in the case of corneas. Therefore, opening new avenues of organ donation where there would be no decision to terminate life-support

systems will not only provide new sources for organs, but in all probability would also increase the current retrieval rate.

This leads to the third problem, expanding the procurement base into the nonheartbeating cadaver population. The moment an organ donor's heart stops beating, the cessation of blood flow results in ischemia. The onset of ischemia initiates a phase of metabolic depression leading to cell death. We know that within 60 minutes of warm ischemia necrosis of the proximal convoluted tubules occurs. The historic approach to organ preservation involves using hypothermia to reduce metabolic activity. However, hypothermic preservation is not benign; it results in edema, alterations in permeability, and tubular damage. The principal difference between ischemia at warm and cold temperatures is the rate at which the cell injury and death occur. Therefore, warm ischemic damage represents the major obstacle to substantially expanding the organ donor pool into the nonheartbeating cadaver population. Organs damaged by warm ischemia cannot tolerate further damage mediated by the hypothermia. Until the damaging effects of ischemia can be alleviated, the donor pool cannot be substantially expanded.

METABOLIC ASPECTS OF IN VITRO ORGAN PRESERVATION

There are seven major parameters involved in the in vitro preservation of organs: 1) ischemia, 2) the effects of the mandatory hypothermia, 3) oxygen consumption in hypothermically preserved organs, 4) ATP synthesis, 5)

inhibition of the ion pumps, 6) alterations in permeability leading to edema and 7) reperfusion injury.

Ischemia

Ischemia, or the cessation of blood flow, will cause the phenomenon of no reflow, which is the failure of the circulation to return. Ischemic-mediated damage is most severe in the first and third segments of the proximal convoluted tubules and this damage is directly related to the length of the ischemia.(12) The initial effects of ischemia are from the lack of molecular oxygen for oxidative phosphorylation; which leads to the depletion of ATP stores within the mitochondria.(13) Nucleotides are rapidly lost during ischemia and this loss is an important factor in the failure of tissue subjected to prolonged ischemia to regenerate ATP after the restoration of the blood supply.(14)

Hypothermia

Currently, all preservation technology is dependant upon hypothermia to diminish the effects of ischemia. The benefits of hypothermia were recognized early, when in 1937 Bickford and Winton noted that hypothermia prolonged the duration of tissue survival.(15) Hypothermia exerts its beneficial effect by diminishing the oxygen demand of the organs and also by reducing the metabolic rate. Normal oxygen consumption by the kidney is approximately 6.3 ml/min.(16) This oxygen consumption is reduced to about half at 30°C and to less than 5% at 4°C.(17) Most organs are stored at temperatures ranging from 4 - 10°C. Similarly, below 22°C a cessation of glomerular filtration occurs and below 18°C tubular activity is inhibited. Most enzyme systems functioning at

normothermia show an approximate two-fold decrease for every ten degrees decrease in temperature.

However, the side-effects of hypothermia are not benign. Cold-induced damage entails organ swelling, loss of endothelial cell integrity, acute tubular necrosis, inhibition of the ion pumps and intracellular acidosis. In fact, hypothermia may be the rate-limiting factor in organ preservation. To control this cold-induced damage, all clinical perfusates employ a variety of impermeants and colloids to control cell swelling.

Oxygen Consumption

Providing an adequate supply of oxygen to the organs was a major obstacle to success in early organ preservation. Oxygen consumption in the kidney is high and this oxygen consumption correlates with renal transport processes. Hypothermia, while reducing metabolism and oxygen consumption, also blocks the effective utilization of oxygen by tissues. At normal physiologic temperatures, the phospholipids making up the cell membranes are highly fluid. Under the hypothermic conditions utilized in organ preservation, the lipid bilayer experiences a phase-change and becomes gel-like, with greatly reduced fluidity. This essentially frozen lipid in the cell membranes negates the utilization of oxygen, even in the presence of a high oxygen-tension. Without the required oxygen, the metabolic consequence for preserved organs is glycolysis.(18)

ATP

Most ATP is synthesized in mitochondria via oxidative phosphorylation.(19) The mitochondria utilize oxygen and

substrate to covert ADP to ATP and in the process reduce oxygen to H_2O. This controlled reduction requires the addition of four electrons. The cytochrome oxidase complex accomplishes this in one step. In doing the reduction in one step, toxic free radical intermediates are not generated. These toxic free radical intermediates include the superoxide anion radical, hydrogen peroxide and hydroxyl radical. Mitochondria normally maintain efficient control systems or scavengers which reduce the small amount of these intermediates generated under normal conditions.

Ischemia whether warm or cold initiate a rapid fall in cellular ATP levels.(20) ATP can be readily resynthesized from adenosine once oxidative phosphorylation resumes at normothermia. Without oxidative phosphorylation, glycolysis is twenty times less efficient than oxidative phosphorylation.(21) The salvage pathway of ATP production produces reactive oxygen species in the process of metabolizing hypoxanthine to xanthine and xanthine to uric acid by means of xanthine dehydrogenase.(22) The depletion of ATP causes an inhibition of the Na pump, active Ca^{++} extrusion stops, fatty acid accumulates and degraded phospholipids are not regenerated. Acidosis develops because the protons released during the synthesis of ATP cannot be converted to H_2O by normal oxidative metabolism.

Ion Pumps

The major impact of ATP depletion is the inhibition of the ion pumps, in particular, the sodium pump. The sodium pump is responsible for maintaining the

intracellular balance of sodium and potassium, and for normal cell volume regulation. The pump exchanges sodium for external potassium. The lack of ATP to drive the pumps results in increased intracellular sodium, more than there being a fall in potassium. The vascular endothelial cells can then swell to double their thickness very quickly. This swelling leads to alterations in permeability resulting in leaky endothelium. If the supply of energy is reestablished before the death of the cells occurs, the process can be reversed and cell volume returns to normal.(23)

Edema

Therefore, the preservation of membrane integrity is probably the major fundamental issue in organ preservation. In all cases where metabolism is inhibited, the result is edema due to increased intracellular H_2O content. The development of leaky endothelium leads to a reduction in blood flow in the medulla which leads to a secondary necrosis of the tubules, which then leads to obstruction and a reduction of glomerular filtration, urine flow and urine concentrating capacity. Therefore, the damage to the endothelium plays a major role in the subsequent renal damage secondary to the preservation.(24)

Reperfusion Injury

Cold preservation followed by rewarming leads to reperfusion injury. Reperfusion injury following hypothermia is a well established concept and its main focus is on the endothelium.(25) Toxic free radical intermediates initiate an injury cascade involving cellular derangement, leukocyte/platelet adhesion and

hypercoagulation. Various scavengers and pharmacologic agents have been used, such as SOD and catalase, calcium antagonists such as chlorpromazine, and prostacyclin and its analog, with various degrees of success. It would be of benefit to avoid the generation of these radicals rather than to attempt to eliminate them. It is apparent that the degree of preservation/reperfusion injury is the direct result of the duration of the cold preservation and not the reperfusion; because reperfusion after short periods of cold ischemia does not lead to graft injury.(26) The extent of the free radical production is also related to the length of the cold preservation. Likewise, blood cell adhesion is directly related to the preservation damage subsequent to the hypothermia.(27)

Hypothermia is the essential foundation of the current technology used in organ preservation. All recent progress in organ preservation can be traced directly to the maneuvers used to control the very damage caused by the hypothermia itself; namely using impermeants and colloids to control cell swelling, pharmacologic agents to stop nucleotide waste, and limiting reperfusion injury while maintaining the membrane integrity.

ORGAN PRESERVATION

Cryopreservation

It has long been the perspective in transplantation that cryopreservation would represent the optimal approach for long-term organ preservation. However, relatively little progress has been achieved in this area in the past 36 years. The first work in cryopreservation was performed by Audrey Smith in 1957; where the freezing and thawing of hamster hearts was attempted without success.(28) In

1967 Halasz and associates attempted cryopreservation of dog kidneys with glycerol or propylene glycol.(29) The kidneys were cooled to -50°C and stored frozen at that temperature for 15 minutes before rewarming. The function of the kidneys after just 15 minutes of freezing was severely impaired. Only brief periods at subzero temperatures have been achieved. The formation of ice crystals within the vasculature appears to be a major problem in whole organ freezing.

Cold Continuous Pumping versus Simple Cold Storage

With the continuing limitations of cryopreservation, extreme hypothermia has represented the cornerstone of organ preservation. There are two primary techniques for organ preservation using hypothermia which were introduced in the 1960's and have been used clinically with virtually no change since that time. The first to be introduced was continuous hypothermic perfusion by Belzer in 1967.(30) In 1969 Collins introduced the concept of simple cold storage.(31) Continuous perfusion allows for oxygenation of the grafts, removal of toxic end-products, in vitro evaluation of flow dynamics and constant maintenance of pH. However it is more expensive and technically more demanding. For longer preservation times or for organs experiencing warm ischemic injury, continuous perfusion is generally considered to be beneficial. The majority of kidneys are now routinely preserved by simple cold storage after flushing with an iced solution, because of its ease of utilization and the minimal cost involved. Despite these two techniques having been in use since the 1960's, the time period of successful organ preservation has not been significantly extended beyond that of the early preservation times of nearly 30 years ago.

DEVELOPMENTAL HISTORY OF ORGAN PERFUSATES

The First Generation of Perfusates

In 1849, Loebell and associates were the first to perfuse isolated kidneys with defibrinated blood.(32) Many others tried perfusing with whole blood, none successfully. These early failures were probably due to hemolysis of the perfusate and the formation of thrombi in the kidneys. Hemodilution with balanced salt solutions were tried next, and these experiments were also found to be damaging.(33) The common problems from all of these experiments were vasospasm, and inadequate flow and pressures. Next cell-free perfusates consisting of plasma were used. However, obstruction of the vasculature continued to be a problem. The hypothermia used in these studies caused the lipoproteins to freeze and precipitate. Belzer contributed an improved perfusate consisting of cryoprecipitated plasma which removed most of the unstable lipoproteins.(34) The precipitated plasma eliminated intravascular lipid deposition and provided the best preservation of kidneys at that time. These early perfusates resulted in problems with stability and lot-to-lot variation. Albumin was found to be less traumatic and this finding lead to new perfusates consisting of semi-synthetic products based upon albumin products.

Second Generation of Perfusates

Serum albumin in various forms was used exclusively for colloid support in these early perfusates. The next generation of perfusates used various synthetics for colloid support, many of these agents are listed in Table I. Keeler first described the phenomenon of a rapid loss

TABLE I

Control of Oncotic Pressure with Colloid

HES	PEG
Pluronics	HSA
BSA	Plasmaproteins
Dextrans	PVP
Ficoll	Gum Arabic
Haemacel	

of intracellular cations during cold preservation in 1966.(35) He found that a potassium rich solution prevented this loss and appeared to be superior to electrolyte solutions used for intravenous infusion. Many studies comparing extracellular-like versus intracellular-like solutions have been undertaken. Overall, the experimental evidence favors the intracellular-like solutions. These intracellular-like solutions were thought to reduce the exchange of intracellular constituents with the extracellular space during preservation. The most notable of these early perfusates were the Collins, Sacks and Eurocollins solutions.

The first Collins solution developed in 1969, was a solution high in potassium and magnesium with glucose, and was phosphate buffered.(36) The rationale for this solution was that it mimicked intracellular fluid, although the magnesium concentration (30mM) was higher than normal free intracellular magnesium which is approximately 1-2mM. The glucose served as a source of impermeant and may have been the primary reason for its

TABLE II

Control of Osmotic Support with Impermeants

Glucose	Sucrose
Mannitol	Raffinose
Gluconate	Lactobionate

success. It soon became apparent that one of the most important properties of an effective perfusate was the presence of impermeants which reduced cell swelling during hypothermic storage. The more common sources of impermeants used in perfusates are listed in Table II.

The Collins, Sacks and Eurocollins solutions were widely used clinically. Mannitol is less permeant than dextrose and has been substituted for it in Collins-like solutions. Sucrose was felt to be better than dextrose for sodium-based perfusates, probably due to glucose uptake via the sodium-glucose co-transporter. Sacks and Eurocollins solutions, similar to the original Collins solution, were intracellular-like solutions high in potassium. The Eurotransplant organization agreed on a standardization preservation solution in 1976.(37) They chose a Collins solution which omitted magnesium sulfate due to problems with it precipitating, and had a higher concentration of dextrose and a higher osmolarity. They named the solution Eurocollins.

Third Generation of Perfusates

The third generation of perfusates, most notably the UW solution or ViaSpan™, are totally synthetic solutions devoid of all animal protein. ViaSpan™ uses HES to

avoid toxicity. There are eleven ingredients in the ViaSpan™ solution: phosphate buffer - to prevent acidosis, adenosine - a precursor for ATP synthesis, it also has vasodilating properties and is a platelet inhibitor, magnesium - cofactor for cation-dependent events, allopurinol - a xanthine oxidase inhibitor to block oxygen radical production, glutathione -to assist in handling oxidative stress and for its reducing capabilities during lipid peroxidation, which may be important during reperfusion, HES - a colloid to prevent expansion of the extracellular space, raffinose - provides osmotic support, and lactobionate - a major organic impermeant anion, since it does not permeate the membrane and therefore, prevents cell swelling. It is also an intracellular-like solution, high in potassium.

It is of interest to note that replacing potassium ions with sodium ions in the ViaSpan™ solution, does not affect the quality of the preservation and some reports describe improved results, particularly in liver transplantation.(38) ViaSpan™ is superior to previous perfusates and generally represents state-of-the-art organ preservation. However, many researchers have questioned the effectiveness of some of the ingredients. There is general agreement that the lactobionate is required, while only one study found HES to be required.

There are now several offshoots of the ViaSpan™ solution, including the HTK, HP16 and Cardisol solutions. These solutions use haemacel or PEG to replace the HES, other sugars to replace the raffinose and other impermeants to replace the lactobionate.(39,40)

THE FUTURE OF ORGAN PROCUREMENT

It is clear that the existing organ donor pool must somehow be expanded. Using the existing pool of heartbeating cadavers, one approach will be to develop more aggressive education programs to increase community awareness. Presumed consent laws may eventually be passed. However four states have turned down such laws, and only one state, Texas, has a modified presumed consent law which permits organ donation if the next-of-kin cannot be found. It is unlikely presumed consent laws will be passed in the U.S. any time in the near future. Offering financial compensation to families for organ donation has the best chance of raising the existing procurement rate from its current rate of only 16.5%. However, our society has a strong bias against the sale of organs.

If we are to expand the donor base into the nonheartbeating cadaver population, a different approach to organ preservation is needed. Warm ischemic damage represents the major obstacle to utilizing nonheartbeating cadavers and similarly, warm ischemic damaged organs cannot tolerate a second insult of hypothermic damage. Interestingly, many of the preservation related problems of severe hypothermia would be eliminated at a more moderate level of hypothermia. Future preservation may be in the range of 18-25°C, where membrane lipids are in a more normal fluid state. Most enzyme systems functioning at 37°C also function at temperatures as low as 20°C, but at a slower rate. More moderate hypothermia would help to: prevent toxic free radicals rather than using scavengers at the time of

reperfusion, eliminate vasospasm, support better oxygen utilization and raise the metabolic rate during preservation. Concordant with using an expanded organ donor pool of nonheartbeating cadavers, there will be a need to develop in vitro parameters of graft viability. Currently the only means of determining graft function is to transplant. And there will probably be a need for organ specific perfusates, designed to support a higher level of metabolism during preservation.

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ENCAPSULATED HEMOGLOBIN: CURRENT ISSUES AND FUTURE GOALS

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ABSTRACT

The promise of encapsulation systems for the sequestration of hemoglobin has been the long-held belief that encapsulation more closely mimics nature's strategy for circulating hemoglobin, and could alleviate hemoglobin based toxicities and increase circulation persistence. Various polymers have been proposed to deliver hemoglobin. One approach toward the encapsulation of hemoglobin has been to employ biodegradable, biocompatible vehicles such as phospholipid vesicles, or liposomes. The majority of encapsulation work with hemoglobin over recent years has focused on liposome encapsulated hemoglobin with demonstrations of efficacy and safety in total and partial isovolemic and hypovolemic exchange models, hemodynamics, circulation persistence and organ biodistribution, processing methods, long term storage through freeze-drying, and serum changes and histopathological consequences following administration in small animals. The data collected thus far indicate that encapsulation of hemoglobin does significantly alter many of the traditionally observed effects following the administration of cell free hemoglobin solutions. Liposome encapsulated hemoglobin circulates for 20-24 hours in small animals and principally

distributes to the liver and spleen. The significant accumulation of liposome encapsulated hemoglobin in these organs poses new questions for short and long-term effects on the reticuloendothelial system and macrophage function which are currently being addressed. In addition, transient hemodynamic and serum changes have been observed following the administration of liposome encapsulated hemoglobin. Many of these are similar to the effects observed following administration of liposomes without intravesicular hemoglobin and are dictated by liposome parameters such as surface charge and character, size, and lipid composition. Finally, fundamental large scale production issues such as encapsulation efficiency and particle size distribution must be optimized to facilitate the commercial development of encapsulated hemoglobin. These issues are discussed in the historical context of encapsulated hemoglobin and basic liposome research, current research status, and future challenges for the development of encapsulated hemoglobin as an artificial oxygen carrying fluid.

Introduction

It is widely accepted that the initial concept of encapsulating hemoglobin can be attributed to seminal work by TMS Chang in that late 1950's [1]. Even before this significant contribution, the toxicities of unmodified hemoglobin were widely known, and thus the desire to mimic the red cell and sequester hemoglobin was undoubtedly considered. Chang first suggested the use of various polymers such as pyroxylin, cellulose nitrate, polystyrene, polyamides and nylon to encapsulate hemoglobin [1,2]. Many of these polymeric systems have not been further developed. The majority of work on encapsulation of hemoglobin has involved the use of biodegradable capsules of lipid called liposomes.

The development of liposomes as potential carriers for hemoglobin followed the work of Alec Bangham in the 1960's which defined the ability to use isolated phospholipids to construct biodegradable capsules which he named

liposomes [3]. The majority of early work on liposomes centered on using liposomes as models to understand biomembrane structure and function and involved studies of permeability, membrane fusion, and phase properties of phospholipids [4]. This work evolved toward applications which examined liposomes as vehicles for the controlled, targeted release of biological agents such as antibiotics, antifungals, antitumoricidal, and cosmetic products [5]. The success of these products has been mixed, largely due to the unrealized goal of efficient targeting of liposomes to specific sites in the body due to the non-specific removal of liposomes by the reticuloendothelial system.

The use of liposomes for the delivery of hemoglobin is a somewhat non-traditional use of liposomes as **release** vehicles, as the application requires the sustained entrapment of hemoglobin with the cooperative uptake and delivery of oxygen. In fact, much attention has been paid to the stability of liposomes to retain hemoglobin intravesicularly due to the known and unknown toxicities of cell free hemoglobin in the vascular compartment and in the kidney. The stability of liposomes in this regard is correlated to the bilayer physicochemical properties and the interactions with serum proteins and cellular components [6,7]. Injected liposomes are quickly coated with IgG, albumin, and opsonins, although the identification of these latter proteins and their role in directing removal of liposomes from circulation has been elusive [6-8]. Liposome components such as cholesterol or polymerizable lipids increase the deformability of the bilayer and decrease the likelihood of rupture from shear forces as the liposomes circulate [9,10]. Other liposome features such as surface charge and character, and chemical makeup also effect the leakage of liposome components following *in vivo* administration [11-20]. Such surface components as ganglioside GM_1, polyethylene glycol derivatives, dextran, and chitin have been explored as modified liposome surfaces to stabilize liposomes *in vivo* [21-23]. The physicochemical properties of

liposomes are also essential in determining the circulation persistence and biodistribution of liposomes.

Circulation Persistence and Organ Biodistribution

One of the original tenants of developing an encapsulated hemoglobin based blood substitute was to extend the circulation persistence as unencapsulated hemoglobin is rapidly cleared from the vascular compartment. The circulation persistence for liposomes is dependent on size, surface charge, surface character, and dose [11-19]. The removal of liposomes from the circulation and permeability of liposomes is largely dominated by the adsorption of serum proteins to the liposome surface [6-8]. Large liposomes (>1.0 microns) are cleared rapidly, while very small diameters (<0.05 microns) persist for longer periods. In addition, liposomes with positively charged surfaces are removed more rapidly than neutral or negatively charged liposomes. It is also widely observed that administration of large doses or pre-dosing of liposomes increases persistence due to saturation of the reticuloendothelial system [12,15]. There has been a large effort in the liposome community to modify liposome surfaces to increase circulation persistence [20-23]. Early efforts focused on the use of ganglioside GM_1 which created a liposome surface rich in monosialic acid groups [22]. GM_1 containing liposomes did increase the circulation persistence of specific liposome formulations. More recently, polyethyleneglycol derivatives of phospholipids have been developed which extend the circulation persistence of liposomes and have been named "stealth" liposomes [23].

The kinetics of circulation persistence observed for liposome encapsulated hemoglobin (at doses of 1.0 g. total phospholipid/Kg and 2.0 g Hb/Kg animal) show a rapid early removal period up to four hours after administration [24-26,27,27b]. It is important to point out that at no time

during liposome administration are the liposomes observed to leak hemoglobin [27,27b]. After the rapid removal phase, there is a slower removal of particles. These complex kinetics are often expressed somewhat misleadingly with a single calculation of circulation half-life. The circulation half-lives reported for the variety of liposome encapsulated hemoglobin formulations explored in small animals vary from 15-24 hours [24-27,27b,33,35,47]. Our group at the Naval Research Laboratory has explored the use GM_1 in liposome encapsulated hemoglobin preparations and observed no increase in circulation persistence in a normovolemic mouse model at similar doses [28]. Other groups are currently exploring the use of polyethylene glycol derivatives to extend the circulation persistence of liposome encapsulated hemoglobin.

The same features which drive the kinetics of circulation persistence also are observed to influence organ biodistribution. Large liposomes are rapidly removed by the lung and liver with smaller liposomes observed to also distribute to the spleen and bone marrow [11]. Tissue resident macrophages and cells of the monocyte phagocytic system are largely responsible for the removal and metabolism of liposomes [9,11,20]. The organ biodistribution of liposome encapsulated hemoglobin have determined that the liver and spleen are the principal sites of accumulation [24,27,27b]. At 20 hours post administration in a normovolemic rabbit, 30% of the injected dose was found in the liver and spleen, 2% in the kidney, with 50% remaining in circulation [27,27b]. Clearance through the liver and spleen has been observed to take place over the course of 1-2 weeks [29]. More specific pathological findings following the administration of liposome encapsulated hemoglobin over this time course will be the focus of a future manuscript by our group (manuscript submitted). The consequence of the significant distribution to the liver is a transient increase in serum transaminases (alanine and aspartate aminotransferase) over the course of 24 hours [29,30]. Enzymes related to

biliary tree function (gamma glutamyl transferase and alkaline phosphatase) were not observed to increase at any time following injection. A more comprehensive examination of serum changes out to two weeks in rats following administration of liposome encapsulated hemoglobin is the focus of a current study (manuscript submitted).

Reticuloendothelial system effects

The accumulation of liposome encapsulated hemoglobin in the liver and spleen has raised new questions regarding the function of the reticuloendothelial system following administration. One study has examined liver function by challenging the liver with carbon particles following varying doses of liposome encapsulated hemoglobin [31]. These results indicated that carbon clearance was decreased at 2 and 12 hours post-treatment (at 1.0 g phospholipid and 2.0 grams Hb/Kg rat) with recovery of baseline values at 24 hours. Kupffer cell phagosomal motion was decreased over this same time course but was returned to baseline values and was returned to baseline at two weeks post injection [31]. More recently, the effect of liposome encapsulated hemoglobin in inducing cultured macrophages to produce tumor necrosis factor, and respond to lipopolysaccharide once exposed to liposome encapsulated hemoglobin has been examined by our group (manuscript in preparation) and others [32]. These results indicate that liposome encapsulated hemoglobin does not induce production of tumor necrosis factor. However, cultured alveolar and peritoneal macrophages, as well as elicited human peripheral blood monocytes when pre-incubated with liposome encapsulated hemoglobin showed reduced tumor necrosis factor response to lipopolysaccharide [32]. Our group has also begun to examine the macrophage mediated cytokine response to liposome encapsulated hemoglobin. In addition, we have previously examined the effect of liposome encapsulated hemoglobin in a mixed lymphocyte reaction, which

showed that leukocytes removed from animals post administration of liposome encapsulated hemoglobin were not impaired in their ability to respond to a challenge from irradiated heterologous lymphocytes [30]. These results are just the beginning of what is a new and important area of investigation into macrophage function, cytokine response, and immunocompetence following administration of liposome encapsulated hemoglobin.

Exchange Transfusion and Top Loading

The data collected to date on the hemodynamics and exchange transfusion of encapsulated hemoglobin can be separated into hemodynamic studies of top loaded material injected into normovolemic animals and total isovolemic exchange transfusions. The first demonstrations of efficacy in total isovolemic exchange transfusion were done by Djordjevich et al [24,33,41]. Their studies clearly demonstrated the ability of liposome encapsulated hemoglobin to transport oxygen *in vivo* and support life below a lethal hematocrit of 2% and have been supported by other groups with different liposome preparations [10,25,34,35].

More recently, a number of transient effects (15-60 minutes) have been observed following the injection of liposome encapsulated hemoglobin which include leukocytosis, hypertension, tachycardia, hemoconcentration, elevation of TXB_2, and thrombocytopenia [36]. The thrombocytopenia observed with liposome encapsulated hemoglobin is observed following the administration of liposomes without hemoglobin and has been attributed to a transient sequestration in the liver and spleen with subsequent rerelease [37]. Some of these effects were alleviated by the substitution of synthetic phosphocholines for the soy based phosphocholine which had measurable lysolecithin contamination [38]. An antagonist for platelet activating factor (BN 50739) also alleviated many of these untoward effects [39]. Partial 50% exchange

transfusion with liposome encapsulated hemoglobin has also been recently examined hemodynamically which documented stable mean arterial pressure in contrast to injection of a saline control which showed hypotension over the same 3 hour observation period [40]. The maintenance of mean arterial pressure was as a result of opposing effects of increased total peripheral resistance and decreased cardiac output. The only previous hemodynamic study of exchange transfusion with liposome encapsulated hemoglobin also showed maintenance of mean arterial pressure. This was ascribed however to an increased cardiac output and vasodilation [41].

Manufacturing Methods and Storage Stability

Early methods to fabricate liposome encapsulated hemoglobin relied on standard liposome preparation procedures such as dispersion in aqueous media followed by sonication, reverse phase evaporation, and detergent dialysis [24,25]. Other liposome preparation techniques including hydrodynamic shear and detergent dialysis have been shown to be amenable to large scale production of encapsulated hemoglobin [43,44]. More recently, a double emulsion/evaporation method have reported higher encapsulation efficiencies (nearly 80%) of the precursor hemoglobin solution [35]. Methemoglobin generation can be minimized by reducing processing temperatures and including soluble and lipid based antioxidants such as glutathione and alpha tocopherol [35,43,44].

In vitro stability of liposome encapsulated hemoglobin is determined by the liposome formulation. One of the reasons charged lipids were included in the formulation for encapsulated hemoglobin was to aid in dispersing the particles and increase the encapsulation efficiency during manufacture [26,43]. The charge surface also aids in preventing aggregation of liposomes in solution. Most liposome formulations used to encapsulate hemoglobin have included

considerable mole fractions of cholesterol which also results in vitro stabilization and little loss of intravesicular hemoglobin with time in liquid storage [45].

There has been considerable effort to increase the shelf-life of liposome encapsulated hemoglobin through freezing or lyophilization [47-49]. These efforts are related to the slow oxidation of the encapsulated hemoglobin in solution as opposed to the disruption of bilayer integrity through liposome fusion or aggregation. The ability to stabilize liposome encapsulated hemoglobin to freezing or lyophilization has been accomplished by including cryoprotectants such as disaccharides and polymerizable lipids [46-49]. The ability to lyophilize a blood substitute is an especially attractive feature for military applications.

Future Challenges

There are a number of important challenges which face the demonstration of safety and efficacy of encapsulated hemoglobin. Many of these are similar to those faced by other hemoglobin-based blood substitutes. The alleviation of hemoglobin induced vasoactivity by encapsulation is an important research goal. Models should address direct comparisons of stroma-free and encapsulated hemoglobin and their interactions with the microvasculature and EDRF.

The continued effort to understand the interaction of encapsulated hemoglobin and the reticuloendothelial system is also important considering the significant accumulation of material in reticuloendothelial organs. Surface modification of encapsulated hemoglobin should focus on increasing circulation persistence to minimize effects of the reticuloendothelial system. The cytokine response to encapsulated hemoglobin and the functional capacity of the reticuloendothelial system following administration of encapsulated hemoglobin

should be explored in models which address hemorrhagic and septic shock would be particularly important in this regard. The understanding of the interaction of hemoglobin and the outer surface of the liposome during manufacture could be an important determinant of these interactions as well as interactions with the vascular bed. The interaction of hemoglobin with the bilayer to produce other potentially deleterious reactions has been studied [49,50] and should continue to be investigated in the context of in vivo response and changes in components with storage.

Finally, alternate encapsulation strategies and production methods should be explored which address further increasing hemoglobin encapsulation efficiency. Controlling particle size distribution (which may also be determined by the propensity of hemoglobin to adhere to the outer surface of the liposome) to allow sterile filtration is also an important goal which will facilitate commercial development of encapsulated hemoglobin products.

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Oxford Research International Corp., Clifton, New Jersey

September, 1992

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DAVID M. COCCHETTO
Glaxo Inc., Research Triangle Park, North Carolina

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edited by
SAMUEL Z. GOLDHABER
Harvard Medical School and Brigham and Women's Hospital, Boston, Massachusetts

October, 1992 / 632 pages, illustrated / $95.00

(Fundamental and Clinical Cardiology Series/12)*

Prepublication praise...

"...During the last few years our concept of venous thromboembolism has changed dramatically....a variety of effective prophylactic and therapeutic strategies have been developed. By conceiving, organizing, editing, and writing large portions of ***Prevention of Venous Thromboembolism***, Dr. Goldhaber has made a singular contribution. This comprehensive up-to-date review of every important aspect of this condition should be of interest to a broad range of medical professionals....

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- reviews common misconceptions that make some physicians reluctant to prescribe prevention
- provides a basis for estimating the cost of diagnosis and treatment versus the cost of prevention
- describes the use of ultra-sonography for making noninvasive diagnosis of DVT and examines processes that mimic its clinical presentation
- offers highly detailed discussions of **when** and **how** to use pharmacological prophylactics such as dextran, heparin, warfarin, low-molecular-weight heparins, and the promising dermatan sulfate
- illustrates the use of nonpharmacological measures, including vascular compression therapy and the latest percutaneous vena caval filters
- compares the risk of VTE when using regional anesthesia and general anesthesia

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Prevention of Venous Thromboembolism
(continued)

- clearly assesses the use of pharmacological, mechanical, and combined procedures for the prevention of VTE among gynecological, coronary, cancer, orthopedic, and neurosurgical patients
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Profusely illustrated and extensively referenced with over **1650** literature citations, 108 tables, and 123 figures, ***Prevention of Venous Thromboembolism*** is a unique resource, indispensable to cardiologists and cardiovascular surgeons; general surgeons; pulmonologists; gynecologists and obstetricians; internists; hematologists; radiologists; pathologists; hospital administrators involved with health care policy; hospital nurses; and medical students, interns, residents, and fellows in these disciplines.

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edited by

SUSANNA CUNNINGHAM-RUNDLES
The New York Hospital-Cornell University Medical Center
New York, New York

September, 1992
576 pages, illustrated
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edited by

ADRIANNE BENDICH
Hoffmann-La Roche, Inc., Nutley, New Jersey

C. E. BUTTERWORTH, JR.
University of Alabama at Birmingham

1991
504 pages, illustrated
$145.00

Micronutrients in Health and in Disease Prevention describes the most widely accepted current research linking essential dietary micronutrients to critical health issues—including cancer, cardiovascular disease, arthritis, impaired immunity, birth defects, cataracts, and other acute and chronic illnesses.

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Pharmacology and Clinical Applications

(Infectious Disease and Therapy Series/8)*

edited by

HAROLD C. NEU
Columbia University College of Physicians and Surgeons
New York, New York

LOWELL S. YOUNG
Kuzell Institute for Arthritis and Infectious Diseases
San Francisco, California

STEPHEN H. ZINNER
Brown University, Rhode Island Hospital, and
Roger Williams Medical Center
Providence, Rhode Island

February, 1993
256 pages, illustrated
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This timely reference addresses the important aspects of the chemistry of the macrolides, azalides, and streptogramins; illustrates how the compounds are related; and details what structural changes are essential to achieve improved antibacterial activity and pharmacology.

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- contains clinical trial findings of eight macrolides used to treat significant infections in children
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edited by
HAROLD P. ADAMS, JR.
University of Iowa, College of Medicine, Iowa City, Iowa

March, 1993 / 728 pages, illustrated / $195.00

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edited by

JEROME S. BRODY and
DAVID M. CENTER
Boston University School of Medicine, Massachusetts

VSEVOLOD A. TKACHUK
Cardiology Research Center, Moscow, Russia

March, 1993
704 pages, illustrated
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PETER F. COHN
State University of New York Health Sciences Center, Stony Brook

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(Fundamental and Clinical Cardiology Series/10)*

edited by
ERIC R. BATES
University of Michigan Medical School, Ann Arbor

September, 1992
528 pages, illustrated
$99.75

This timely reference offers in-depth coverage of **all** pharmacological *and* mechanical interventions available for the treatment of acute myocardial infarction.

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